Characterisation of Hydrocarbon Degrading Antarctic *Pseudomonas* species

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Abstract

Hydrocarbon contamination of Antarctica poses a threat to the unique and fragile ecosystems of this continent. Bioremediation, utilising indigenous hydrocarbon-degrading bacteria, has been proposed as an environmentally friendly method for clean up of contaminated terrestrial sites in Antarctica. Design and implementation of a successful bioremediation process requires detailed information of the degradative bacteria present at the contaminated site(s). This study focused on toluene and naphthalene-degrading *Pseudomonas* species isolated from JP8 jet-fuel contaminated sites at Scott Base, Antarctica.

Isolates were assessed for their ability to grow on toluene and *meta*-toluate or naphthalene as a sole carbon source on solid and in liquid media at 6°C and 15°C. All the toluene degrading isolates could grow on 5 mM *meta*-toluate in liquid and on solid media at 6°C and 15°C. Toluene degrading isolates, with the exception of 8/43 and 8/51, were able to grow on solid media supplemented with toluene vapour but none could grow on toluene in liquid media. Naphthalene degrading isolates could grow on solid media, with naphthalene vapour as a carbon source, at 15°C but at 6°C two of the six isolates, 8/47 and 7/38, could not grow on solid media supplemented with naphthalene vapour. None of the toluene and naphthalene degrading isolate could grow on carbon-free solid or liquid media without the addition of a growth substrate. Presumptive plasmids were isolated from the following toluene degrading isolates, 8/46, 7/163, 7/167, 7/22, and the following naphthalene degrading isolates, 7/156, 7/38, ant5, ant9. Two toluene degrading isolates, 7/167 and 8/46, were studied in more depth. Both isolates grew in liquid media, with *meta*-toluate supplied as a sole carbon source, over a range of temperatures, 6°C, 10°C, 15°C, 20°C, and 25°C. The optimum growth temperature for isolate 7/167 is between 20°C and 25°C and the optimum growth temperature for isolate 8/46 is 15°C. Isolates were able to sustain growth on *meta*-toluate at 6°C, 15°C, and 25°C. Isolate 7/167 could not sustain growth on *meta*-toluate at 30°C. Isolate 8/46 could sustain growth on *meta*-toluate at 30°C but could not grow at 35°C. All of the Antarctic isolates are psychrotrophic.

Degradative plasmids were isolated from 7/167 and 8/46. Restriction enzyme digest fragment patterns of plasmid DNA generated with XhoI showed isolates 7/167 and 8/46 contain different sized plasmids, 61 kb and 95 kb, respectively. Regions of toluene catabolic genes were amplified by the Polymerase Chain Reaction. Sequence data of the PCR products produced using *xylB* primers revealed isolate 7/167 to be 100% homologous to the *xylB* gene region amplified from the archetypal TOL plasmid, pWWO, isolated from the Northern Hemisphere, and 8/46 to be 92% homologous.

Assays of four enzymes, involved in toluene degradation, catechol 1,2-dioxygenase, catechol 2,3-dioxygenase, benzyl alcohol dehydrogenase, and benzaldehyde dehydrogenase, from isolates 7/167 and 8/46 showed catechol 2,3-dioxygenase, benzyl alcohol dehydrogenase, and benzaldehyde dehydrogenase to be present and active. No activity was detected for catechol 1,2-dioxygenase, indicating the presence of a *meta* cleavage pathway of toluene degradation, typical of that found on TOL type plasmids, and not *ortho* cleavage of catechol typical of chromosomal gene expression.

Isolates 7/167 and 8/46 contain large plasmids, similar to Northern Hemisphere isolated TOL degradative plasmids, and cleave *meta*-toluate via the *meta* cleavage pathway. This degradative mechanism is analogous to the toluene degradative pathway encoded by the archetypal TOL plasmid, pWWO, in the mesophilic bacterium *P. putida* mt-2. These indigenous psychrotrophic toluene degrading Antarctic bacteria have the ability to degrade *meta*-toluate at temperatures typical of the Antarctic soil at Scott Base in the summer months. These toluene degrading Antarctic bacteria have the potential to be used to develop a bioremediation programme to successfully clean up JP8 jet fuel contaminated soil at Scott Base, Antarctica.

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Chapter 1

Introduction and Literature Review

INTRODUCTION

Antarctica is a unique environment and its' indigenous biota are susceptible to human impacts. One of the major sources of human impact is the pollution of Antarctic sites by fuel. JP8 jet-fuel is the commonest fuel used in Antarctic-Ca. This fuel is comprised of low molecular weight hydrocarbons, such as alkanes (C8-C15) and toluene and naphthalene, and is used for heating, and to run generators and vehicles, at the scientific bases and temporary field camps. Hydrocarbon contamination occurs when fuels are stored and handled. Under the protocol on Environmental Protection to the Antarctic Treaty, New Zealand has a responsibility to protect this environment and associated ecosystems and minimise the impact of humans on this continent. In accordance with this Act, fuel (hydrocarbon) contaminated sites in Antarctic need to be decontaminated. Bioremediation has been proposed as a method for decontamination of these sites. Bioremediation employs the use of microbes to metabolise the contaminant to harmless by-products. As the introduction of foreign organisms is prohibited in Antarctica, indigenous hydrocarbon-degrading microbes must be use to clean up hydrocarbon contaminated Antarctic sites. In order to design and implement a successful bioremediation programme, to clean up hydrocarbon contaminated sites, more information is needed about aromatic hydrocarbon degrading Antarctic bacteria. This thesis aims to provide some of the required information.

The aims of this thesis are threefold;

1. Determine if toluene degrading Antarctic isolates can grow and sustain growth on toluene and *meta*-toluate over the range of temperatures typical of Antarctic summer soil temperatures.
2. Isolate degradative plasmids from Antarctic isolates and analyse them for size and presence of degradative genes, in order to determine the type of toluene degradative pathway present in Antarctic bacteria.

3. Assess the activity of toluene degradative enzymes in toluene degrading Antarctic isolates to further elucidate the degradative pathway utilised by these bacteria.

LITERATURE REVIEW

1.1 Fuel (Hydrocarbon) Pollution in Antarctica

Antarctica is the fifth largest continent on earth and makes up 10% of the worlds land surface and 10% of its' oceans. The Antarctic environment is unique primarily due to extreme cold and aridity, extreme seasonal variations in light, over 98% ice-cover, geographic isolation, and limited human impact. The uniqueness of this environment has allowed sets of organisms to evolve and adapt to the abiotic environment (Benninghoff and Bonner, 1985; Franzmann, 1996). A number of Antarctic bacterial species show lower optimum growth temperatures compared to non-Antarctic species, for example, Antarctic *Methanococcoides burtonii* has a optimum growth temperature of 23°C whereas the non-Antarctic species has a temperature optimum of 35°C (Franzmann, 1996). Thus Antarctic organisms may be very sensitive to environmental changes beyond the narrow range of changes occurring naturally in the environment (Benninghoff and Bonner, 1985).

The major human activity that takes place on the Antarctic continent is scientific research conducted by various nations (Myers *et al*, 1980), although, tourism in Antarctica is fast becoming significant, with tourist numbers doubling over the last decade to 9604 in the 1997/98 summer season (Antarctica New Zealand Annual Report, 1997/98). The permanent bases where scientific research is conducted in Antarctica tend to be located on the ice-free coastal regions (Cripps and Priddle, 1991). These ice-free coastal regions have conditions that favour the establishment of biological communities (Benninghoff and Bonner, 1985) and thus these fragile biological communities are vulnerable to the impacts of human activity (Myers *et al*, 1980, Benninghoff and Bonner, 1985).

Fuels (hydrocarbons), used to support the scientific research bases in Antarctica, are the most wide spread pollutants in Antarctica (McDonald *et al*, 1992). The majority of fuel contaminated sites are situated at past and present permanent research bases and

temporary camps (Cripps and Priddle, 1991). Fuel spillage is associated with handling and storage of fuels (Cripps and Priddle, 1991) and fuel contamination has proved lethal to the marine life and the limited terrestrial biota of Antarctica (Kennicutt, 1990; Cripps and Priddle, 1991; Eppley, 1992). Studies have been conducted on the effect of fuel contamination on Antarctic ecosystems and biota. A documented example was the *Bahia Paraiso* oil spill near Palmer Station on the Antarctic Peninsula in 1989, where more than 150,000 gallons of fuel was released into the surroundings bays (Kennicutt, 1990). The effect of fuel contamination resulted in the deaths of marine and bird life in the area (Kennicutt, 1990; Eppley, 1992). In most cases fuel contamination due to human activity is restricted to research bases and field camps, with spillage represents the major source of pollution (Cripps and Priddle, 1991), and may impact on the immobile plant and microbial communities colonising these areas.

Under the protocol on Environmental Protection to the Antarctic Treaty (1991) New Zealand has a responsibility to protect the Antarctic environment and associated ecosystems and to minimise the impact of humans in Antarctica (Antarctica New Zealand Information Bulletin, 1996). Fuel (hydrocarbon) contamination impacts on the Antarctic environment. The major fuel used at Scott Base, Antarctica is JP8 jet-fuel. JP8 jet-fuel is used to for heating, to run vehicles and generators, and to fuel portable field generators. This fuel consists for low molecular weight *n*-alkanes (C8-C15) and aromatic hydrocarbons such as toluene and naphthalene. JP8 is comprised of approximately 18% aromatic hydrocarbons, with naphathlenes making up 0.67% (JET A-1 Product Quality Report). These low molecular weight hydrocarbons tend to be less resistant to degradation than high molecular weight polycyclic aromatic hydrocarbons (Cerniglia, 1992).

A number of methods exist to clean up fuel contaminated sites but some remediation methods are not appropriate for implementation in Antarctica. In order to clean up fuel contaminated sites in Antarctica development of a bioremediation program has been proposed. Bioremediation technologies utilise microbes to metabolise the contaminant to harmless by-product thereby decontaminating the site. Due to the prohibition of the introduction of foreign organisms to Antarctica, indigenous hydrocarbon degrading organisms must be used to develop a bioremediation program in Antarctica (Antarctic

Conservation Act, 1978, sect 670-4, cited in Tumeo and Guinn, 1997). Indigenous hydrocarbon-degrading bacteria have been isolated from fuel contaminated soils near Mawson Base (Kerry, 1990), at Jubany Scientific Station on King George Island (MacCormack and Fraile, 1997), at McMurdo Station (Tumeo and Wolk, 1994), Scott Base, Marble Point, and Lake Vanda in the Ross Dependency (Aislabie *et al*, 1998). The bacteria studied in this thesis have been isolated from JP8 jet-fuel contaminated sites at Scott Base, Antarctica, and are able to degrade toluene or naphthalene. This study investigates aspects of indigenous Antarctic hydrocarbon degrading bacteria genetics, metabolism and growth as part of assessment for developing a successful bioremediation programme to clean up hydrocarbon contaminated soils at Scott Base, Antarctica.

1.2 Methods of Clean up of Fuel Contaminated Sites

A number of methods for decontamination of fuel polluted sites have been developed. Many of these methods are not cost effective or publicly acceptable and are inappropriate for implementation in Antarctica.

Landfill disposal, which involves excavation and disposal of contaminated material, including soil, can be costly, depending on land values, and merely moves the contaminated soil from one site to another without removing the contaminant (Wilson and Jones, 1993; McNicoll and Baweja, 1995).

Incineration of contaminated soil is a technically complex treatment process and is very costly, can be up to ten times the cost of bioremediation, (U.S.EPA, 1998). This process is also publicly unacceptable (U.S.EPA, 1998). Solvent or detergent washing techniques to remove the contaminant results in the waste products still having to be detoxified either chemically or biologically (Singleton, 1994). This remediation method is technically complex and can be costly.

Stabilisation of the contaminant in soil involves using chemical and physical methods to immobilise the contaminant in the soil. This technology is expensive and again

does not remove the contamination. There is little information on the long-term stability of the contaminant in the soil, thus these treated soils may become unusable in the future (McNicoll and Baweja, 1995).

In contrast the use of bioremediation technologies to clean up contaminated sites is economical, once implemented, and is seen as environmentally friendly and is less disruptive to the environment than other remediation technologies (McNicoll and Baweja, 1995).

1.2.1 Bioremediation of Fuel Contaminated Sites

Bioremediation of fuel contaminated sites employs the use of microbes to metabolise the contaminant to non-hazardous products such as CO_2 and cell biomass. The microbial metabolism (degradation) of aromatic hydrocarbons, as an energy and carbon source has been intensively studied in an effort to develop technologies to remove these toxic compounds from contaminated environments (Bartha and Atlas 1977; Dagley, 1978, Leahy and Colwell, 1990; Smith, 1990; Cerniglia, 1993). A successful bioremediation strategy involves either encouraging the native microbial community to degrade the pollutant more rapidly, or addition of effective degradative microbes in the absence of native degrading microbes (Prince, 1992). Biodegradation of hydrocarbons is principally an aerobic process, but anaerobic degradation also occurs (Prince, 1992). Various abiotic factors in the contaminated site can affect the ability of microbes to degrade the contaminant ie. temperature, pH, water and oxygen availability, salinity, toxicity and concentration of the contaminant, and nutrient availability (nitrogen and phosphate) (Fewson, 1988; Leahy and Colwell, 1990).

The most publicised use of bioremediation was the clean-up of oil contaminated beaches after the *Exxon Valdez* oil spill in Prince William Sound, Alaska, in 1989 (Pritchard and Costa, 1991). The addition of fertilisers to oiled beaches overcame the nitrogen and phosphorous limitations. As a result the indigenous degradative microbial populations were enriched and the biodegradation of the oil was enhanced, resulting in the oil removal from these beaches (Pritchard and Costa, 1991).

Bioremediation would be the most appropriate process for clean up of hydrocarbon contaminated Antarctic soils due to the presence of indigenous hydrocarbon-degrading bacteria (Kerry, 1990; Aislabie, 1997), the environmentally friendly nature of the process and the low technical complexity of the process, and low cost once implemented.

1.2.2 Types of Bioremediation

A number of bioremediation technologies have been developed and implemented which differ depending on the nature of the contaminated site and the contaminant.

In situ bioremediation technologies treat the contaminated soil in place, thus have the advantage for being less disruptive to the site. *In situ* treatment includes both surface and sub-surface remediation (U.S.EPA, 1991). Contaminants in the surface soil can be degraded by encouraging the microbial degradative processes via tilling to aerate the soil and spray irrigation to add growth limiting nutrients (U.S.EPA, 1991). Contaminated sub-surface soil, where oxygen and nutrients maybe limiting, can be treated by bioventing to optimise the soil for degradative bacterial metabolism and growth (U.S.EPA, 1998). Instillation of air injection wells enables the contaminated area to be supplied with microbial growth limiting factors (U.S.EPA, 1998).

Ex situ bioremediation involves treatment of contaminated soils within a bioreactor or by landfarming. Landfarming involves excavating contaminated soil and thinly spreading it over an impermeable surface. The soil is tilled in order to aerate it and promote volatilisation and microbial degradation of the contaminant. This technology is relatively cheap but does require considerable land area (McNicoll and Baweja, 1995). Treatments that utilise a bioreactor vary but in all cases use of a bioreactor enhances the microbial biodegradation process by controlling the oxygen and nutrient concentrations, and optimising the temperature and pH (U.S.EPA, 1991). Treatments using a bioreactor vary according to the type and location of the contaminant. Composting involves aeration of a highly biodegradable material, like woodchips, with a small percentage of biodegradable contaminated material. Slurry-phase treatment combines contaminated soil with water to form slurry. Nutrients and oxygen is added

to the bioreactor as needed and the resulting clean soil, once separated from the water, and is able to be returned to the environment (U.S.EPA, 1991). Solid-phase treatment of contaminated soil involves placement of the soil in a lined treatment bed. Nutrients, water, and oxygen are added to aid microbial biodegradation of the contaminant.

In all cases the presence of bacteria able to degrade the pollutant must first be ascertained, then selection of and optimisation of a bioremediation technology can be achieved (U.S.EPA, 1998). *In situ* bioremediation technologies would likely be the most appropriate for implementation in Antarctica. Kerry (1993) showed that addition of nutrients to hydrocarbon contaminated surface soils in the Vestfold Hills in Antarctic resulted in an increase in microbial activity and a decrease in hydrocarbon concentration at these sites.

1.3 Biodegradative Bacteria

Hydrocarbon degrading bacteria and fungi are widely distributed in marine, fresh water, and soil habitats (Atlas and Bartha, 1992). The most prevalent hydrocarbon degrading bacteria in the soil environment are *Pseudomonas, Arthrobacter, Micrococcus, Nocardia, Vibrio, Acinetobacter, Rhodococcus, Corynebacterium, Sphingomonas, and Mycobacterium* (Bartha and Atlas, 1977; Atlas and Bartha, 1992; Korda *et al*, 1997).

1.3.1 Degradative Pseudomonads

Pseudomonas species are typical aerobic soil bacteria and this genus consists of a number of fluorescent and non-fluorescent species. No thermophilic species have been found although some psychrotrophic species and strains are known (Auling, 1993; Ray *et al*, 1994; Gumley and Inniss, 1996; Whyte *et al*, 1996; Chablin *et al*, 1997; Kannan *et al*, 1998). Pseudomonads are chemo-organotrophic gram negative, motile rods (Brock *et al*, 1994) and belong to the family Pseudomonadaceae and the class Proteobacteria (Auling, 1993).

Pseudomonads occur in many different habitats. Their nutritional requirements are simple whereas their metabolic versatility is extreme. Pseudomonads play a significant role in the global carbon cycle through metabolism of various organic molecules including aromatic compounds (Auling, 1993). The metabolic versatility of pseudomonads derives in part from the presence of extrachromosomal elements called plasmids. Plasmids are harboured in many *Pseudomonas* species and strains (Auling, 1993). Plasmids can encode genes for the metabolism or degradation of a wide range of simple organic compounds, as well as hydrocarbons and synthetic compounds (Frantz and Chakrabarty, 1986). It is noteworthy that degradative genes can be chromosomally encoded and that similar degradative genes can be located on the chromosome in one organism and located on the plasmid in another organism (Harayama and Timmiss, 1989).

1.3.2 Antarctic Pseudomonads

Degradative bacteria have been isolated from Antarctic soils and like other continents *Pseudomonas* species are a common feature of the degradative bacterial population in Antarctica.

Pseudomonas species are rare in Antarctic soils (Boyd and Boyd, 1962; Johnson *et al*, 1978; Wyn-Williams, 1990; Vishniac, 1993). However, *Pseudomonas* species have been isolated from soils at Cape Royds, Cape Evans, Marble Point (Boyd and Boyd, 1962), McMurdo Station (Johnson *et al*, 1978; Wyn-Williams, 1990), and Scott Base (Aislabie, 1997) in the Ross Dependency, Scirmacher Oasis, Queen Maud Land (Shivaji *et al*, 1989), the Vestfold Hills and Mawson Base (Line, 1988, Kerry, 1990), and Jubany Station (King George Island, South Shetland Islands) (MacCormack and Fraile, 1997).

A number of hydrocarbon degrading *Pseudomonas* species have been isolated from oil contaminated Antarctic soils (Kerry, 1990; Aislabie, 1997; MacCormack and Fraile, 1997) and Kerry (1990) and Aislabie (1997) found that numbers of Antarctic hydrocarbon degrading bacteria were enhanced in fuel contaminated Antarctic soils compared to pristine Antarctic soils.

Nothing is known about the degradative genes or degradative mechanisms used by hydrocarbon degrading Antarctic *Pseudomonas*. Research on degradative *Pseudomonas* isolated from other continents shows that degradative genes tend to be located on plasmids (Auling, 1993). Plasmids have also been found in degradative *Pseudomonas* isolated in other extreme cold regions ie. Baffin Island (Whyte *et al*, 1997) and Resolute Bay area of the North West Territories, Canada (Whyte *et al*, 1996). Shivaji *et al*, (1994) have isolated plasmids from a number of Antarctic bacterial isolates belonging to the genera *Pseudomonas*, *Arthrobacter*, *Sphingobacterium*, *Planococcus*, and *Micrococcus*.

As little is known about the degradative genes and mechanisms of hydrocarbon degradation by Antarctic *Pseudomonas* species, this review will focus on research on degradative *Pseudomonas* in other regions of the world. In accordance with this research topic only the toluene and naphthalene degradative pathways will be reviewed.

1.4 Toluene Degradation by Pseudomonads

The genes for toluene metabolism in pseudomonads can be plasmid or chromosomally encoded. A number of plasmids specify the degradation of toluene, xylene, and their derivatives. These plasmids differ in size, transmissibility, and restriction endonuclease fragment patterns (Duggleby *et al*, 1977). A number of different toluene and xylene degradative pathways exist (Worsley and Williams, 1975; Deutz *et al*, 1994; Williams and Sayers, 1994; Shields *et al*, 1995). Five unique bacterial catabolic pathways that result in the degradation of toluene have been described. I will review the two most well studied pathways, the TOL pathway and the *tod* pathway, and briefly comment on the other three pathways.

1.4.1 TOL Toluene Degradative Pathway

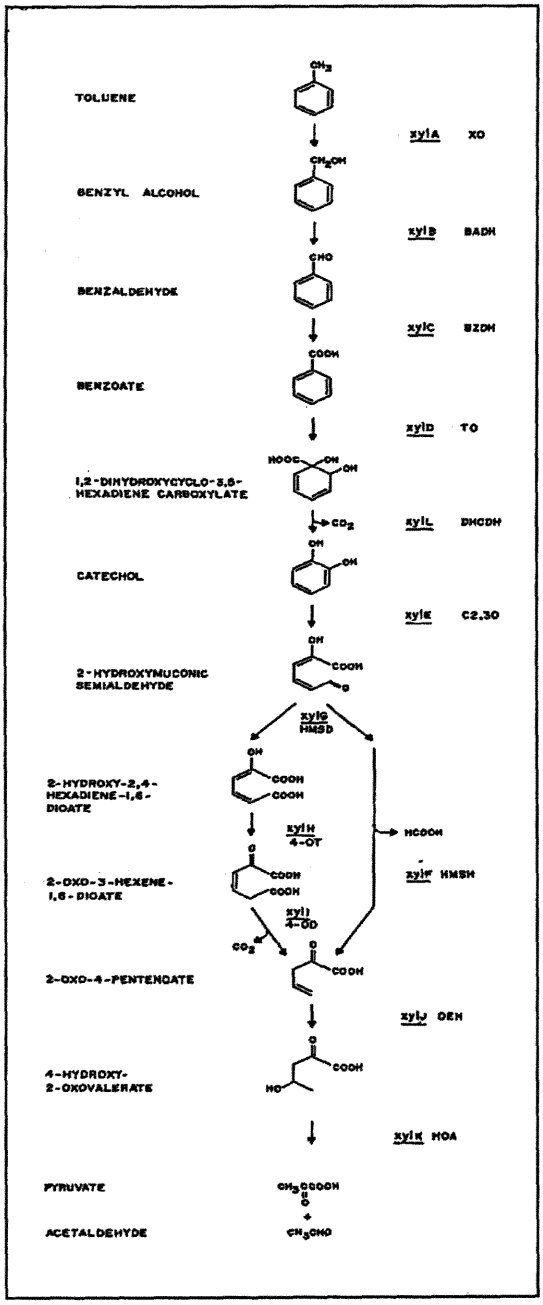
The TOL plasmid represents a group of plasmids that specify the degradation of *m*-xylene, *p*-xylene, toluene, and their corresponding alcohol and acid derivatives through the *meta* pathway to acetaldehyde and pyruvate (Worsley and Williams, 1975; Frantz

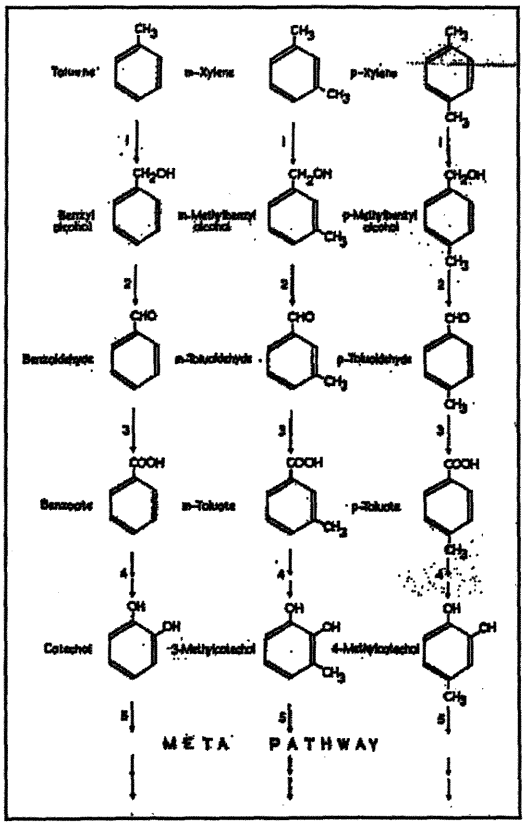
and Chakrabarty, 1986). This is the commonest route of toluene degradation by pseudomonads (Williams and Sayers, 1994). The most characterised of the TOL toluene-degradative plasmids is the pWWO plasmid in *Pseudomonas putida* (*arvilla*) mt-2. The plasmid is 117 kilobase pairs (kb) in size (Duggleby *et al*, 1977) and approximately 40 kilobase pairs encode the catabolic pathway and regulatory genes (Burlage *et al*, 1989).

Regions of the TOL plasmid are known to undergo spontaneous recombination with the chromosome (Frantz and Chakrabarty, 1986). A 56 kilobase region, including the degradative pathway genes, found on TOL type plasmids has been identified as a transposable element. Genes involved in transposition have been identified and this 56 kilobase region is bound by direct repeats indicating the possibility of transposition and recombination (Frantz and Chakrabarty, 1986). Transposition and recombination occurrence is also indicated by the duplication of the plasmid encoded catechol 2,3-dioxygense gene and the chromosomally encoded catechol 1,2-dioxygenase gene in various strains (Chatfield and Williams, 1986; van der Meer *et al*, 1992). A further indication of the transfer of these catabolic plasmids through a bacterial population is the strong DNA homologies seen among TOL, NAH (naphthalene degradation) and SAL (salicylate degradation) plasmids (Williams and Sayers, 1994). There are also many examples of self-transmissible plasmids that carry genes for degradation of organic compounds (van der Meer *et al*, 1992).

The archetypal TOL toluene degradative plasmid, pWWO, from *Pseudomonas putida* (*arvilla*) mt-2, harbours the *xyl* genes for toluene and xylene degradation (Worsley and Williams, 1975). The *xyl* catabolic genes are organised into two operons, (Figure 1.1) referred to as the upper and lower (*meta*) pathways (Burlage *et al*, 1989).

A number of the degradative genes and enzymes involved in the degradation of toluene and encoded by the TOL pathway have been characterised. Three genes (*xylB, xylC, xylE*) and their gene products, benzyl alcohol dehydrogenase, benzaldehyde dehydrogenase, and catechol 2,3-dioxygenase, respectively, have been researched in this study, will be reviewed, as will *xylA* (codes xylene monooxygenase) the first gene in this TOL degradative pathway.





1= Toluene oxidase, 2= benzyl alcohol dehydrogenase, 3= benzaldehyde dehydrogenase, 4= benzoate oxidase, 5= catechol 2,3-dioxygenase

Gene	Enzyme or function	Abbreviation
xylA	Xylene oxygenase	XO
xylB	Benzyl alcohol dehydrogenase	BADH
xylC	Benzaldehyde dehydrogenase	BZDH
xylD	Toluate oxygenase	TO
xylX	Terminal oxidase?	
xylY	Terminal oxidase?	
xylZ	?	
xylE	Catechol 2,3-oxygenase	C2,3O
xylF	2-Hydroxymuconic semialdehyde hydrolase	HMSH
xylG	2-Hydroxymuconic semialdehyde dehydrogenase	HMSD
xylH	4-Oxalocrotonate isomerase	4-OT
xylI	4-Oxalocrotonate decarboxylase	4-OD
xylJ	2-Oxopent-4-enoate hydratase	OEH
xylK	2-Oxyohydroxypent-4-enodate aldolase	HOA
xylL	Dihydroxycyclohexadiene carboxylate dehydrogenase	DHCDH
xylR	Regulation	
xylS	Regulation	

Figure 1.1. Toluene and xylene degradative pathways encoded by the pWWO plasmid in *Pseudomonas putida* (*arvilla*) mt-2. (Burlage *et al*, 1989, Worsley and Williams, 1975).

1.4.1.1 Upper Operon

The upper operon consists of genes (*xylABC*) encoding enzymes for the oxidation of toluene and xylenes to toluates and benzoates (Figure 1.1). The upper pathway of the TOL plasmid pWW0 encodes three enzymes, xylene monooxygenase (*xylA*), benzyl alcohol dehydrogenase (*xylB*), and benzaldehyde dehydrogenase (*xylC*) (Burlage *et al*, 1989).

Xylene monooxygenase (*xyl*A) is the first enzyme in the TOL toluene metabolic pathway encoded by the plasmid, pWWO. This enzyme catalyses the conversion of toluene to benzyl alcohol by the addition of molecular oxygen (Burlage *et al*, 1989). Xylene monooxygenase consists of two subunits coded for by two genes, *xylA* codes for a 40-KDa protein and *xylM* codes for a 35-KDa protein (Harayama and Timmis, 1989). The second step in the toluene degradative pathway is the conversion of benzyl alcohol to benzaldehyde by benzyl alcohol dehydrogenase (*xylB*). This enzyme is a 40-KDa dimer of identical subunits (Harayama and Timmis, 1989) and is a zinc containing long chain alcohol dehydrogenase (Shaw *et al*, 1993). Benzaldehyde dehydrogenase is the third enzyme in the upper pathway and catalyses the conversion of benzaldehyde to benzoate (Burlage *et al*, 1989). Benzaldehyde dehydrogenase is a 57-KDa protein (Harayama and Timmis, 1989).

1.4.1.2 Lower Operon

The lower operon of the TOL plasmid pWWO from *P. putida (arvilla)* mt-2 is comprised of nine genes (*xylDLEGFJKIH*) (Burlage *et al*, 1989) that code for enzymes that convert upper operon generated compounds to cell biomass and CO_2. The lower (*meta*) pathway genes encode enzymes for the conversion of benzoates and toluates to acetaldehyde and pyruvate via the *meta* ring cleavage of a catechol intermediate (Burlage *et al*, 1989; Harayama and Timmis, 1989; Smith, 1990). Acetaldehyde and pyruvate are then metabolised through the Krebs cycle.

Catechol 2,3-dioxygenase, the enzyme product of the *xylE* gene in the TOL pathway, has been well characterised. This gene is located on the lower pathway of the TOL toluene degradative plasmid, pWW0. Catechol 2,3-dioxygenase consists of four

identical subunits and requires molecular iron for activity (Burlage *et al*, 1989). This enzyme catalyses the incorporation of two atoms of molecular oxygen into catechol. Catechol is cleaved in the *meta* position and 2-hydroxymuconic semialdehyde is formed (Bugg and Winfield, 1998). The archetypal pWW0 plasmid has a single *xylE* gene in contrast to other plasmids that have two *xylE* genes (Chatfield and Williams, 1986).

P. putida mt-2 also contains chromosomally encoded catabolic genes involved in the degradation of catechol, a product of toluene metabolism. These catabolic genes mediate the degradation of catechol via the *ortho* pathway (Burlage, *et al*, 1989). The *ortho* pathway encodes the gene (*catA*) for catechol 1,2-dioxygenase. This enzyme initiates ring cleavage of catechol in the *ortho* position to produce *cis, cis*-muconate, which is subsequently degraded to succinate and acetyl coenzyme A (Chatfield and Williams, 1986; Burlage *et al*, 1989; van der Meers *et al*, 1992).

Control over which degradative pathway is expressed, *meta* or *ortho*, is determined by the substrate to be metabolised (Burlage *et al*, 1989). For example, benzoate and toluene induce the plasmid encoded upper and lower (*meta*) pathway genes and are degraded via this pathway (Kivisaar *et al*, 1989), whereas catechol, though it is an intermediate of toluene metabolism, is metabolised by chromosomally encoded *ortho* pathway gene products (Burlage *et al*, 1989). Two regulatory genes control the expression of the encoded upper and lower (*meta*) degradative pathways of the TOL plasmid, pWWO. Gene expression is controlled at the transcription level (Inouye, *et al*, 1987). Ramos, *et al* (1987) have developed a model of the sequence of reactions that result in the expression of a plasmid encoded toluene degradative pathway in *P. putida* mt-2. The substrate, ie. toluene, enters the cell and binds to a constitutively expressed xylR protein. A NtrA sigma factor then binds to this complex and the entire complex binds to the upper operon promoter. The upper operon genes (*xylABC* and *xylS*) are expressed. The metabolic products created by the upper operon enzymes and the increased levels of xylS protein induce the expression of lower (*meta*) pathway genes and the metabolic products are degraded to pyruvate and acetaldehyde. The XylS protein can induce the expression of the lower degradative pathway in the presence of lower (*meta*) pathway substrates (Inouye *et al*, 1987).

1.4.2 *Tod* Toluene Degradative Pathway

The genes for toluene catabolism in *P. putida* F1 are chromosomally encoded and are located on a single operon (Williams and Sayers, 1994). The first enzyme in the seven-step pathway of toluene degradation is a three-component toluene dioxygenase (*todABC1C2*) (Zylstra and Gibson, 1989). This enzyme converts toluene to *cis*-toluene-2,3-dihydrodiol through the addition of a single diatomic oxygen (Zylstra and Gibson, 1989; Lau *et al*, 1994; Williams and Sayers, 1994; Shields *et al*, 1995). The dihydrodiol is converted to 3-methylcatechol which is then cleaved in the *meta* position by 3-methylcatechol 2,3-dioxygenase (*todE*). The *meta* fission product, 2-hydroxy-6-oxohepta-2,4-dienoate, is hydrolysed and metabolised to Krebs cycle intermediates by three enzymes, xylJ hydratase, xylK aldose, and xylQ AA dehydrogenase (Lau *et al*, 1994).

1.4.3. Other Toluene Degradative Pathways

Three other toluene degradative pathways have been found and as with the TOL and *tod* pathways the oxygenase-catalysed hydroxylation of toluene, by a monooxygenase, the first step of toluene degradation, differs in these pathways. Toluene monooxygenases, that hydroxylate the aromatic ring in all three possible positions, producing *ortho*, *meta*, and *para* cresol, have been described (Shields *et al*, 1995).

TOM, a 108kb degradative plasmid from *Burkholderia (Pseudomonas) cepacia* G4, constitutively expresses a toluene catabolic pathway distinguished by a unique toluene *ortho*-monooxygenase (TOM). The TOM plasmid initiates toluene catabolism by the *ortho* hydroxylation of toluene to produce *ortho*-cresol (Shields *et al*, 1995). *Pseudomonas pickettii* PK01 contains a chromosomally encoded toluene *meta*-monooxygensae that hydroxylates toluene to produce a *meta*-cresol intermediate (Kaphammer *et al*, 1990). The chromosomally encoded toluene *para*-monooxygenase (*tmoABCDE*) of *Pseudomonas mendocina* KR1 initiates toluene degradation by hydroxylation of the substrate in the *para* position to produce *para*-cresol (Whited and Gibson, 1991, van der Meer *et al*, 1992).

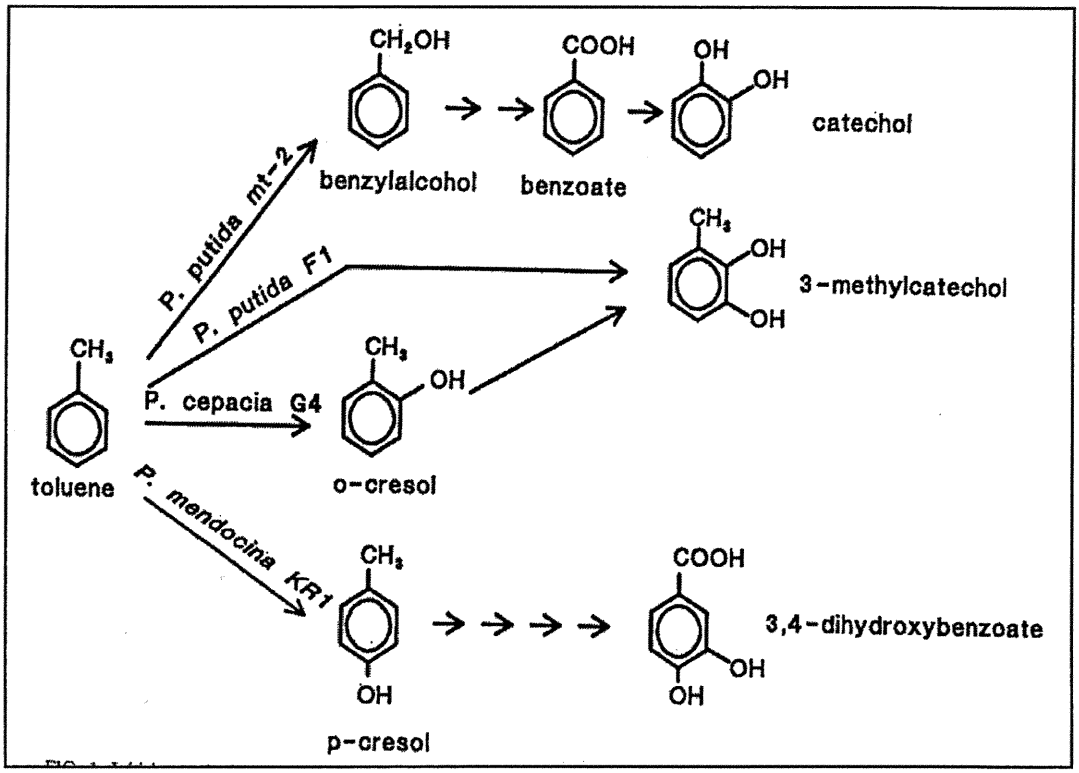


Figure 1.2. Various toluene degradative pathways. (Deutz *et al*, 1994)

1.5 Naphthalene Degradation by Pseudomonads

The NAH group of plasmids are closely related to TOL type plasmids and specify the degradation of naphthalene through the *meta* cleavage pathway (Harayama and Timmis, 1989). NAH and TOL plasmids share considerable DNA sequence homology (Harayama and Timmis, 1989) and the genes specifying the degradation of catechol to pyruvate and acetaldehyde are in the same order on the NAH and TOL plasmids (Harayama and Timmis, 1989; Lau *et al*, 1995).

The archetypal naphthalene catabolic plasmid, NAH7 (83kb), from *P. putida* PpG7 has its' genes organised into two operons (Figure 1.3). The nah1 operon (upper degradative pathway) contains genes (*nahABCDEF*) responsible for the conversion of naphthalene to salicylate. The sal or nah2 operon (lower or *meta* degradative pathway) (*nahGHIJK*) specifies the degradation of salicylate to pyruvate and acetaldehyde (Frantz and Chakrabarty, 1986; Yen and Serdar, 1988; Harayama and Timmis, 1989;

Smith, 1990). The first step in the degradation of naphthalene by *P. putida* PpG7 involves the incorporation of two atoms of oxygen into naphthalene by naphthalene dioxygenase to form *cis*-dihydroxy-1,2-dihydro-naphthalene (Cerniglia, 1984). This product is then converted to 1,2-dihydroxynaphthalene and cleaved by a dioxygenase to yield *cis*-2-hydroxybenalpyruvate. This compound is then converted, via a series of dioxygenases, to salicylate and pyruvate (Cerniglia, 1984). Salicylate is oxidised by salicylate hydroxylase to catechol, which undergoes *meta* ring cleavage by catechol 2, 3-dioxygenase to pyruvate and acetaldehyde (Yen and Gunsalus, 1982).

Induction of the nah operon is positively controlled at the transcription level by a constitutively expressed *nahR* gene in the presence of the inducer, salicylate (Yen and Gunsalus, 1982; Frantz and Chakrabarty, 1986; Lal *et al*, 1995). The *nahR* gene in *P.putida* PpG7 is functionally analogous to the *xylR* gene in toluene catabolism by *P.putida* mt-2 (Yen and Gunsalus, 1982). Naphthalene does not induce the expression of the nah operons in *P. putida* PpG7. In the cell naphthalene is converted to salicylate by low-level constitutively expression of the nah1 operon (upper operon) (Yen and Serdar, 1988). Salicylate induces the nah operons and thus degradative gene expression (Yen and Serdar, 1988)

In some cases the entire naphthalene degradative pathway is encoded by the plasmid and in others the pathway maybe partially plasmid and partially chromosomally encoded (Yen and Gunsalus, 1982; Frantz and Chakrabarty, 1986). The identification of a salicylate degradative plasmid only in a Nah^+ Sal^+ host *P. putida* NP suggests that either the NAH plasmid has yet to be identified in this strain, or that the genes for naphthalene metabolism are located on the chromosome (Yen and Serdar, 1988). Dunn and Gunsalus (1973) showed that *P.putida* PpG7 also contains a chromosomally encoded *ortho* degradation pathway and catechol 1,2-dioxygense enzyme.

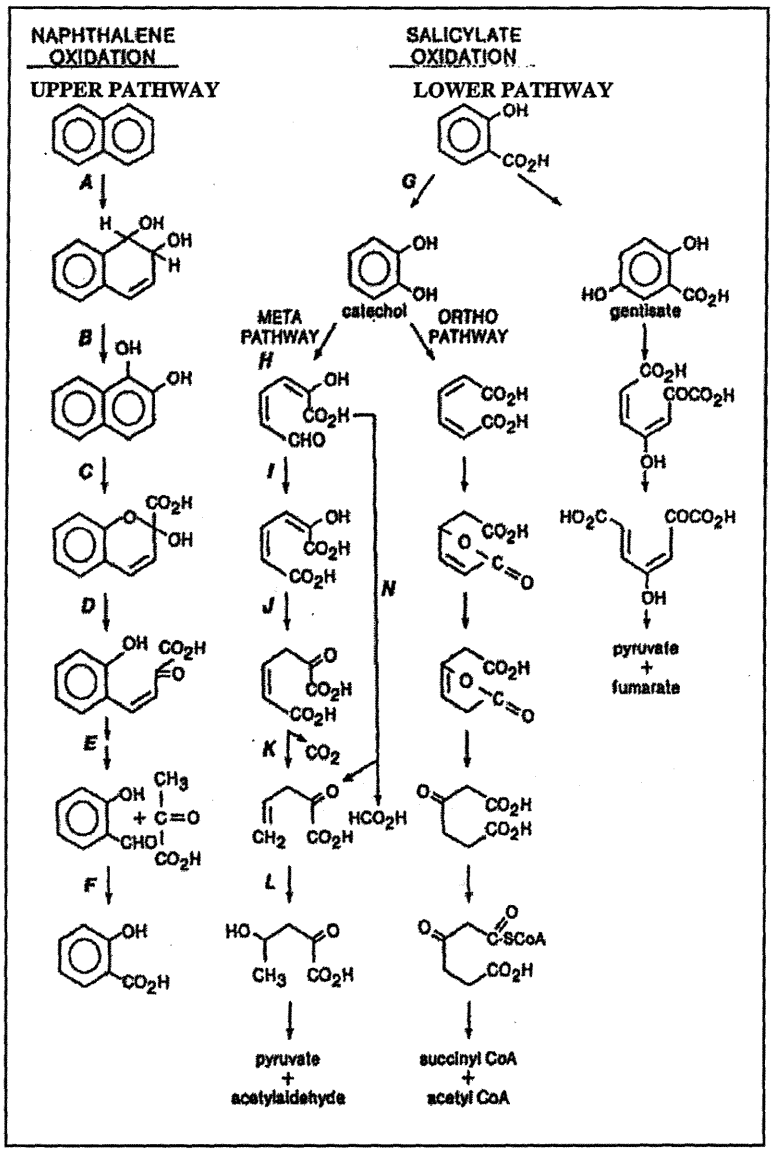


Figure 1.3. Naphthalene degradation pathway encoded by NAH7 plasmid in *Pseudomonas putida* PpG7. (Yen *et al*, 1988). **A**= *nahA*, naphthalene dioxygenase, **B**=*nahB*, cis-naphthalene dihydodiol dehydrogebase, **C**= *nahC*, 1, 2-dihydroxynaphthalene oxidase, **D**= *nahD*, 2-hydroxychromene-2-carboxylate isomerase, **E**= *nahE*, 2-hydroxybenzalpyruvate, **F**= *nahF*, salicyladehyde dehydrogenase, **G**= *nahG*, salicylate hydroxylase, **H**= *nahH*, catechol oxygenase, **I**= *nahI*, 2-hydroxymuconic semialdehyde dehydrogenase, **J**= *nahJ*, 2-hydroxymuconate tautomerase, **K**= *nahK*, 4-oxalocrotonate decarboxylase, **L**= *nahL*, 2-oxo-4-pentenoate hydratase, **N**= *nahN*, 2-hydroxymuconic semialdehyde hydrolase.

Chapter 2.
Materials and Methods

2.1 Source of Antarctic Bacterial Isolates

The toluene and naphthalene degrading bacteria used in this study were isolated from JP8 jet fuel contaminated soils at Scott Base, Antarctica, by Landcare Research scientists (Figure 2.1). Soil samples were collected from a 0-5 cm depth from the site of a fuel leak near the Scott Base kitchen (Figure 2.2), from below a fuel storage tank at Scott Base (Figure 2.3), and from a site of a leaking pipeline carrying JP8 jet fuel from McMurdo Base to Williams Field (Figure 2.4) (Aislabie, 1997). Soils at these sites are mineral soils, have a high pH, and a low water content (Aislabie, 1997).

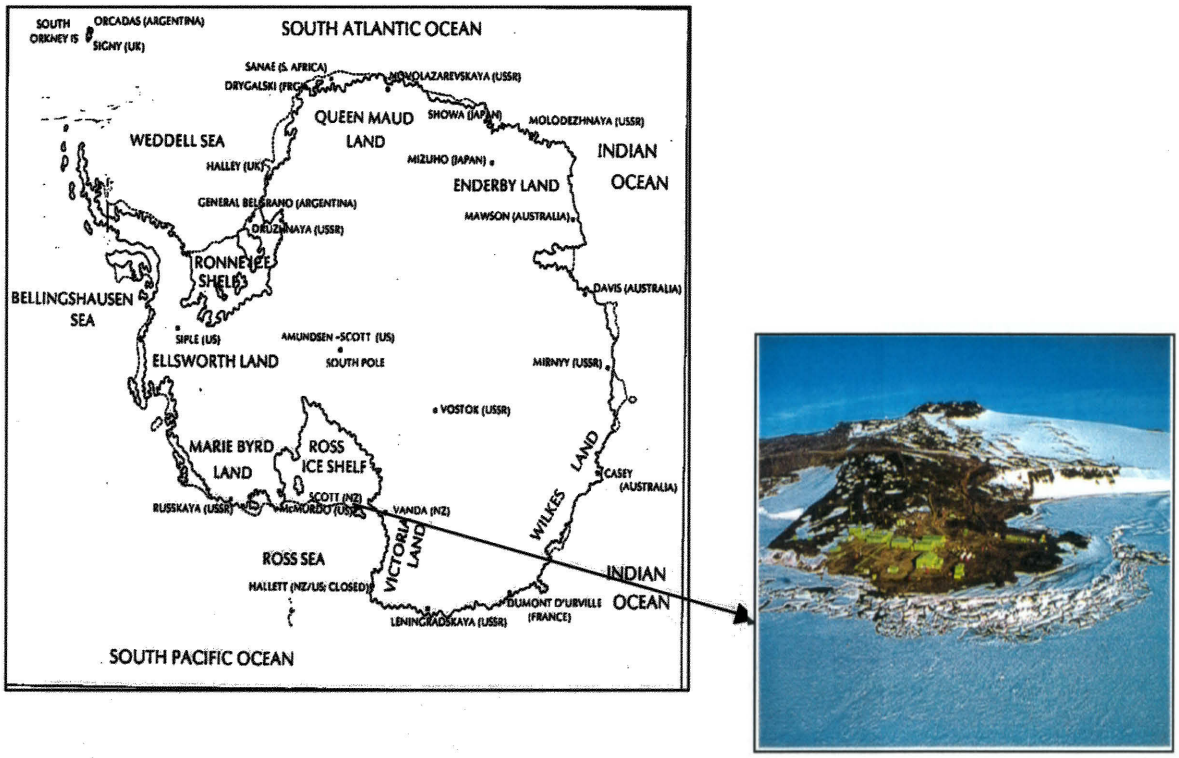


Figure 2.1. Scott Base on the Ross Dependency, Antarctica.

Figure 2.2. Site of a fuel leak near the Scott Base kitchen. Isolates numbered with the prefix 7 were isolated from this site.

Figure 2.3. Site of fuel storage tanks at Scott Base. Isolates ant5 and ant9 were isolated from this site.

Figure 2.4. Site of a leaking pipeline carrying JP8 jet fuel to Williams Field. Isolates numbered with the prefix 8 were isolated from this site.

All bacterial isolates used in this study are listed in table 2.1 and were maintained at -70°C using the Protect system (Technical Service Consultant Ltd., Bury, England).

Table 2.1. Antarctic Bacterial Isolates used in this study.

Antarctic Isolates	Hydrocarbon Degraded
8/46	Toluene/*meta*-toluate
8/43	Toluene/*meta*-toluate
8/51	Toluene/*meta*-toluate
7/163	Toluene/*meta*-toluate
7/167	Toluene/*meta*-toluate
7/22	Toluene/*meta*-toluate
8/47	Naphthalene
7/157	Naphthalene
7/156	Naphthalene
7/38	Naphthalene
ant5	Naphthalene
ant9	Naphthalene

2.2 Bacterial Cultivation Methods

Appendix A lists all growth media used in this study. All media were sterilised by autoclaving at 121°C for 20 minutes.

2.2.1 Toluene Degrading Bacteria

Toluene degrading isolates were routinely grown and maintained on solid nutrient rich R2A media (Difco) and solid selective media. Toluene-degraders were maintained on carbon-free Bushnell Haas Broth (Difco) solidified with 1.6% purified agar (Oxoid) (BHA) and amended with 5mM *meta*-toluate (Aldrich) (BHA-mtol) (Williams and Murray, 1974) or 100 µl of neat toluene (BDH scintillation grade) added as a vapour in the lid of the petri plate. Strains were stored on BHA-mtol and BHA supplemented with toluene vapour and R2A at 15°C for up to four weeks.

Liquid cultures of toluene degrading isolates were grown on either half concentration of nutrient rich Plate Count Broth (Difco) (½ PC) or on Bushnell Haas Broth (Difco) (BH) supplemented with 3 ml of neat toluene in a vapour tube in the growth vessel or 5mM *meta*-toluate dissolved in the liquid media.

Pseudomonas putida mt-2 was maintained and grown on 5 mM *meta*-toluate at 30°C using methodology described for toluene degrading isolates (Williams and Murray, 1974).

2.2.2 Naphthalene Degrading Bacteria

Naphthalene degrading isolates were grown and maintained, similarly to toluene-degraders, on BHA supplemented with 0.1 g naphthalene crystals (M&B) added as a vapour in the lid of the petri plate or on R2A or in ½ PC broth.

2.2.3 Inocula Generation

Inocula were generated by removing 4-5 individual bacterial colonies, of various sizes, from BHA-mtol or BHA supplemented with neat toluene or BHA supplemented with naphthalene crystals, and inoculating 50 ml of ½ PC. Cultures were grown at 15°C with shaking at 200 rpm. Culture density was measured on an Ultraspec 3000 (Pharmacia Biotech) at 660 nm in 3 ml plastic cuvettes. Log phase starter cultures of approximately 10^{10} cells (OD_{660} 2.0-2.5) were used as inoculum for various experiments.

2.3 Isolate Identification

Antarctic isolates were assessed microscopically for morphology and mobility, and checked for their reaction to gram stain (Crabtree and Hinsdill, 1974). A number of the isolates were characterised by use of API 20 NE test strips (bioMerieux).

2.4 Initial Growth Characterisation

2.4.1 Solid Media

The ability of Antarctic isolates to grow on toluene or naphthalene as sole carbon source on solid medium was verified. Bacterial colonies from R2A plates were streaked onto BHA-mtol and BHA supplemented with toluene, or BHA supplemented with naphthalene. Plates were incubated at 6°C and 15°C for 25 days. Carbon-free control BHA were also streaked with bacteria and used for comparison to assess growth.

2.4.2 Liquid Media

Toluene degrading isolates were assessed for growth on liquid media at 6°C and 15°C. BH broth (50 ml), supplemented with *meta*-toluate dissolved in the media or toluene as a vapour, was inoculated with 1ml starter culture and incubated, shaking at 200 rpm, at 6°C and 15°C. The optical density (OD_{660}) was read from time zero of incubation

every 24 hours until stationary growth phase. Carbon-free BH broth controls were inoculated and used for comparison to assess growth.

2.5 Temperature Effect on Growth of Isolates 7/167 and 8/46 on *meta*-toluate and Optimum Growth Temperature Determination

Toluene degrading isolates 7/167 and 8/46 were selected for further in-depth study. Growth of the isolates on *meta*-toluate at 6°C, 10°C, 15°C, 20°C, 25°C, and 30°C was investigated.

Triplicate flasks containing 50 ml of BH amended with 5mM *meta*-toluate were equilibrated to the respective growth temperatures overnight and 1 ml of starter culture was added to each flask. The optical density of each culture flask was read at regular intervals over the period of incubation starting at time of inoculation and preceding to stationary phase. The maximum growth rate at each temperature was calculated from triplicate samples at log phase growth. Inoculated carbon-free BH controls were also assessed for bacterial growth. Isolate 8/46 was able to grow on *meta*-toluate at 30°C and so was assessed for ability to grow on BHA-mtol at 35°C.

The isolates did not grow on toluene vapour in liquid culture growth experiments, so to assess the retention of the isolates' ability to grow on toluene, and culture purity, serial dilutions of each culture at each growth temperature were prepared in phosphate buffer (Appendix B). Triplicate samples of 100 µl of diluent were plated onto BHA-mtol and incubated at 15°C for 10 days or until colony formation was observed. Stabs of 30 individual colonies were taken from BHA-mtol plates with sterile toothpicks and transferred to BHA supplemented with toluene as a vapour. Plates were incubated at 15°C for 10 days or until colony growth was observed. The percentage toluene degraders versus *meta*-toluate degraders was calculated.

2.6 Sustained Growth of Toluene-degrading Isolates 7/167 and 8/46 on *meta*-toluate

The ability of isolates, 7/167 and 8/46, to sustain growth on *meta*-toluate at 6°C, 10°C, 15°C, 25°C, and 30°C, was determined.

Stationary phase cells from the liquid cultures from growth experiment (section 2.5) were harvested by centrifugation at 4000 rpm for 15 minutes at 4°C. The supernatant was decanted and the cell pellet was resuspended in 1 ml of ½ PC. The optical density (OD_{660}) of the resuspended cells was measured. Based on the optical density reading, a volume of cells equal to 1 ml of optical density 3.5 (OD_{660}) was added to fresh BH containing 5 mM *meta*-toluate. If the final volume of cells added was less than 1ml then the volume added was made up to 1 ml with ½ PC. The culture was grown to stationary phase and the process was repeated. Average optical densities and standard deviations were calculated for each growth triplicate.

To confirm the retention of the isolates degradative ability, an aliquot of 100 µl of log phase cells taken from each of the triplicate flasks was plated onto BHA-mtol and BHA supplemented with toluene, and carbon-free control BHA. Plates were incubated at 15°C for 10 days or until colony formation was observed.

The 7/167 and 8/46 cultures grown at 6°C did not obtain a cell density high enough to give an optical density (OD_{660}) of 3.5 so samples were pelleted and resuspended in 1 ml ½ PC and inoculated into fresh BH media amended with 5 mM *meta*-toluate.

Isolates 7/167 and 8/46 were unable to sustain growth at 6°C when studied using the above method. To further assess both isolates ability to sustain growth at 6°C on *meta*-toluate the growth experiment was repeated but cells were not harvested; instead a 1 ml sample of stationary phase cells was taken from the BH growth flask and inoculated directly into fresh BH amended with 5 mM *meta*-toluate.

Isolate 8/46 was able to sustain growth on *meta*-toluate at 30°C and so was assessed for growth on BHA-mtol at 35°C.

2.7 Plasmid Isolation

A number of methods for plasmid isolation were tried on a number of Antarctic isolates (see Appendix C for details), including chromosomal shearing method, electrophoresis well method, and large and small scale isolation by alkaline lysis (Sambrook *et al*, 1989), but plasmids were not successfully isolated using these methods. Partially successful plasmid isolation was achieved using a modification of the protocol of Farrell (1980) and successful plasmid isolation was achieved using the Kieser method (Kieser 1984). These two methods will be described

2.7.1 Farrell Plasmid Isolation Method

The method used was developed by Farrell (1980) and resulted in the isolation of partially purified plasmid DNA. This method involves lysozyme and N-lauroyl sarcosine (Sigma) and sodium chloride mediated bacterial cell lysis and the DNA released is purified by size separation in a salt (cesium chloride) gradient.

Unless specified all solutions were at 4°C and all reactions carried out at 4°C on ice. A starter culture was grown to log phase over night at 15°C shaking at 200 rpm. Triplicate flasks containing 500 ml of ½ PC broth were inoculated with 1 ml of the starter culture and grown to log phase (OD_{660} 2.0-2.5) at 15°C, shaking, overnight. The bacterial cells were harvested by centrifugation at 6000 rpm for 15 minutes. The cell pellet was resuspended in 100 ml of Tris sucrose (50 mM Tris chloride pH 8, and 25% w/v sucrose) and homogenised by shaking vigorously for 2-3 minutes. A freshly prepared 20 ml solution of 5 mg/ml lysozyme (Boehringer Mannheim) was added to the cell suspension and the suspension was held on ice for 20 minutes. A 20 ml volume of 250 mM EDTA was added and the solution incubated on ice for a further 5 minutes. The bacterial cells were lysed by the addition of a room temperature solution of 40 ml N-lauroyl sarcosine (Sigma) and 50 ml of 5 M sodium chloride. The lysis solution was mixed for 1 minute and held on to ice for a further 15 minutes. The lysate was separated from the cell debris by centrifugation at 20,000 rpm for 30 minutes. The supernatant was decanted and the DNA was precipitated at 4°C overnight with ¼ volume of Polyethylene glycol 6000 (BDH). The precipitated DNA was harvested at 14,000 rpm for 8 minutes. A white pellet of DNA was visible in the

centrifuge bottle. The supernatant was decanted and the DNA pellet was air dried at room temperature for 30 minutes. The DNA pellet was then resuspended in Tris-EDTA buffer (TE) pH8 (Appendix B)) and purified further by a cesium chloride gradient.

To separate linear chromosomal DNA and nicked plasmid DNA from covalently closed circular (CCC) plasmid DNA a cesium chloride gradient was created following a modification of the method of Farrell (1980). Cesium chloride (Boehringer Mannheim) was added to the DNA preparation to give a refractive index reading of 1.3995. The DNA was visualised by the addition of Ethidium bromide (10 mg/ml) to give a final concentration of 740 µg/ml (Sambrook *et al*, 1989). The cesium chloride gradient was created by centrifuging of the preparation in a Beckman Ultracentrifuge at 40,000 rpm for 24 hours at 20°C. A TLA 100.3 rotor (Beckman) was used whereas the Farrell method called for a Ti50 rotor.

The resulting gradient should have produced two bands of DNA; in the procedure in this thesis two bands were observed. The upper band consisted of linear chromosomal and nicked plasmid DNA and the lower band consisted of covalently closed circular (CCC) plasmid DNA. The DNA bands were collected into separate tubes using a 25-gauge needle and syringe. Ethidium bromide was removed from the DNA using changes of cesium chloride saturated isopropanol and the cesium chloride was removed by dialysis in two changes of TE pH8 at 4°C over 24 hours. The DNA was further purified by precipitation at –20°C overnight with and two volumes of ice-cold 99.95% ethanol (BDH analytical grade). The DNA was harvested and resuspended in TE pH8 and used for Polymerase Chain Reaction (PCR) and DNA sequencing reactions.

2.7.2 Keiser Plasmid Isolation Method

The plasmid isolation method of Kieser (1984) resulted in the isolation of pure plasmid DNA from isolates 7/167, 8/46, and the positive control isolate *P. putida* (*arvilla*) mt-2. This method uses lysozyme and alkali to lyse the bacterial cell and purification of

plasmid DNA by removal of chromosomal DNA and proteins with an acid phenol/chloroform solution.

A starter culture was grown to log phase over night at 15°C with shaking at 200 rpm. Flasks containing 50 ml of ½ PC broth were inoculated with 1ml of the starter culture and grown to log phase (OD_{660} 2.0-2.5) at 15°C, shaking, overnight. The bacterial cells were harvested by centrifugation at 10,000 rpm for 10 minutes. The cell pellet was resuspended in 20 ml of lysis buffer (0.3 M sucrose, 25 mM Tris chloride at pH 7.5, and 25 mM Na_2EDTA) The cell walls were broken down by the addition of 2 mg/ml lysozyme (Boehringer Mannheim) and subsequent incubated at room temperature for 5 minutes. Cells were then lysed by 10 ml of lysis solution (0.3 M NaOH and 2% sodium dodecyl sulphate) and incubation at 55°C for 20 minutes then cooled to room temperature.

To remove the protein and linear DNA, a 10 ml solution of acidified phenol/chloroform (5 ml chloroform, 5 ml phenol, 1 ml distilled water, and 5 mg 8-hydroxyquinoline) was added to the preparation. The solutions were mixed slowly by inversion to prevent shearing of large plasmid DNA. The aqueous and organic phases were separated by centrifugation at room temperature for 5 minutes at 10,000 rpm. The upper aqueous phase was transferred to a fresh tube, avoiding transfer of any interphase material, and an equal volume of chloroform was added and the phases again separated by centrifugation as described. The upper aqueous phase was transferred to a fresh tube and the DNA was further purified by precipitation overnight at –20°C with 1/10 volume of 3 M sodium acetate pH 7 and two volumes of ice-cold 99.95% ethanol (BDH analytical grade). The precipitated DNA was collected by centrifugation at 10,000 rpm for 30 minutes. The DNA pellet was then rinsed with 70% ethanol and pelleted again by centrifugation as described. The DNA pellet was resuspended in TE buffer pH 8 and used for restriction enzyme digestions and PCR experiments.

DNA collected using the Farrell and Kieser methods described was analysed for presence of plasmid and chromosomal DNA by agarose gel electrophoresis and agarose gel electrophoresis of restriction endonuclease digests of the DNA.

2.8 Restriction Endonuclease Digestion of Plasmid DNA

Plasmid DNA isolated by the Kieser and Farrell methods was analysed for purity and plasmid size by digestion with restriction endonuclease enzymes, SmaI and XhoI.

DNA isolated from Antarctic isolates 7/167 and 8/46 and control DNA isolated from *P. putida* mt-2 was digested with SmaI and XhoI restriction enzymes (Boehringer Mannheim) using a standard method outlined by Sambrook *et al*, 1989. To a final volume of 18 µl, containing 0.2-1 µg of DNA, 2 µl of the appropriate 10X restriction enzyme digestion buffer was added and mixed with the DNA. A volume of 1 µl of restriction enzyme (10 units enzyme per µl) was added to the sample and mixed gently by tapping the tube. The DNA digestion solution was incubated at the appropriate temperature for 3-4 hours and then a further 10 units of enzyme was added. The DNA was then digested overnight for 16 hours at the appropriate temperature.

The digestion fragments produced were analysed by migration patterns generated by agarose gel electrophoresis. The digested DNA (25 µl) was mixed with 2 µl of loading dye (Appendix B) and electrophoresed in Tris-acetate/EDTA electrophoresis buffer (TAE buffer) (Appendix B) on a 1% agarose (SeaKem LE, FMC Bioproducts) gel amended with ethidium bromide. DNA digest fragments were visualised by ultraviolet (UV) illumination of the ethidium bromide. Restriction fragment size, and subsequently plasmid size, was determined by comparison of the migration distance of plasmid restriction digest fragments to Lambda DNA HindIII fragments (Gibco BRL) and 100 base pair DNA ladder (Gibco BRL).

Table 2.2. Restriction enzymes used in this study.

Restriction Enzyme	Sequence Cleaved	Digest Temperature
SmaI	CCC↓GGG	25°C
XhoI	C↓TCGAG	37°C

2.9 Amplification of Toluene Degradative Gene Sequences using the Polymerase Chain Reaction (PCR)

DNA isolated by the Farrell plasmid isolation method from Antarctic isolates 7/167 and 8/46 and the control isolate *P. putida* mt-2 was assessed for the presence of toluene degradative gene sequences by the Polymerase Chain Reaction (PCR).

PCR was used to amplify regions of three degradative genes (*xylE*, *xylB*, *xylC*) (Figure 1.1) in Antarctic isolates 7/167 and 8/46 and *P. putida* mt-2. These three genes are known to be involved in toluene metabolism by *P. putida* mt-2 and thus are possibly involved in the toluene degradative pathway of Antarctic toluene-degrading bacteria.

PCR reaction conditions and *xylE* forward and reverse primer sequences were taken from Whyte *et al* (1996). The *xylE* reverse primer sequence from Whyte *et al* (1996) was discarded after PCR results showed low primer binding specificity to 7/167 and 8/46 DNA. A second *xylE* reverse primer, and *xylB* and *xylC* forward and reverse primers were designed from the DNA sequence of *P. putida* mt-2 TOL plasmid, pWWO (http://www.ncbi.nlm.nih.gov/htbin-post/Entrez/nucleotide.html).

Table 2.3. Polymerase Chain Reaction primers used to amplify degradative gene sequences in the toluene degradative pathway.

Gene Amplified	Primer	Primer Sequence (5'-3')
xylE	*xylE* forward	GTG CAG CTG CGT GTA CTG GAC ATG AGC AAG
	xylE reverse	TTC GTC GCC ATA CAT GAG GGC GTG GTC GAA
xylB	*xylB* forward	GCG ACA TAT GCG CTA TCT
	xylB reverse	GCG TCG CCC CAA GTT CAC T
xylC	*xylC* forward	AAC TAA AGG AGC GCG AGT
	xylC reverse	CTT TAA GAT CAC CGC ATT

Polymerase Chain Reactions were generated using Boehringer Mannheim PCR kits and were optimised on the Eppendorf Mastercycler gradient PCR machine. The standard protocol determined for optimum PCR was:

DNA melting temperature of 94°C for 60 seconds,

Primer annealing temperature of 50°C for 60 seconds,

Elongation temperature of 72°C for 60 seconds.

The PCR cycle was repeated 40 times and completed with a final elongation step of 72°C for 6 minutes.

The amplified DNA (20 µl) was mixed with 2 µl of loading dye and electrophoresed in TAE buffer on a 1% agarose (SeaKem LE, FMC Bioproducts) gel amended with ethidium bromide. PCR products were visualised by UV illumination of the ethidium bromide. The pWWO plasmid DNA from *P. putida* mt-2 was used as a positive control to determine that the primers and PCR was working.

Table 2.4. Components of Polymerase Chain Reaction Mix

PCR Components	Reaction Volume
10x PCR Reaction Buffer ($MgCl_2$)	5µl
200 µM of each deoxynucleoside triphosphate (dATP, dTTP, dGTP, dCTP)	5µl
0.2 µM Forward Primer	1µl
0.2 µM Reverse Primer	1µl
Taq Polymerase (1U/µl)	1µl
DNA (10ng)	2µl
Sterile distilled water	35µl

2.10 Degradative Enzyme Assays

Isolates 7/167 and 8/46 were assayed for the presence of four enzymes involved in toluene degradation (Figure 1.1). Two ring cleavage enzymes, catechol 2,3-dioxygenase (*meta*-cleavage) and catechol 1,2-dioxygenase (*ortho*-cleavage) were assayed based on the protocols of Nozaki (1970) and Hollender *et al* (1990), respectively. Benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase were assayed according to the protocol of Worsley and Williams (1975). To ensure detection of any enzymes activities over the duration of culture log phase growth, cell-free extracts were prepared from early log phase cell growth (28 hours incubation at 15°C) and from late log phase cell growth (50 hours incubation at 15°C) grown on 5mM *meta*-toluate (Figure 3.3 and 3.4) and assayed.

2.10.1 Enzyme Extraction Method

Duplicate flasks containing 100 ml BH amended with 5 mM *meta*-toluate were inoculated with 1 ml of an overnight culture. The cultures were grown at 15°C shaking at 200 rpm to early log phase of culture growth (28 hours incubation). Cells were harvested by centrifugation at 6000 rpm for 5 minutes at 4°C. The pellet was washed twice with phosphate buffer (Appendix B). The washed pellet was resuspended in 50 mM phosphate buffer pH 7.5 containing 10% acetone to give 0.5 g wet weight/ml. The cell suspension was lysed by sonication on ice three times for 30 seconds with holding on ice for a minute between sonications. Cell debris was removed by centrifugation at 10,000 rpm for 15 minutes. The supernatant containing the cell-free extract was decanted and used for enzyme assays. This procedure was repeated with cells being harvested at late log phase of growth (50 hours incubation). All enzyme extracts were held on ice throughout the duration of the experiment, except when assayed. All assays were performed at room temperature on an Ultraspec 3000 spectrophotometer (Pharmacia Biotech).

All assays were performed in quartz cuvettes and all enzyme assays were performed in duplicate. All substrate solutions for assays were prepared fresh on each occasion. Controls consisting of 700 µl substrate and 70 µl of boiled cell-free extract were also assayed for activity of each enzyme tested.

Catechol 2,3-dioxygenase activities were calculated using the catechol molar extinction coefficient 36000 $M^{-1}cm^{-1}$ (Shepherd, 1997).

Benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase activities were calculated using the Nicotinamide-adenine dinucleotide (NAD^+) molar extinction coefficient, 63000 $M^{-1}cm^{-1}$ (Boehringer Mannheim Information sheet). Cell numbers were calculated by conversion of optical density (OD_{660}) readings to cell dry weight and then to cell number (Ingraham *et al*, 1983). Enzyme activities are expressed as µmoles of substrate transformed per minute per cell and calculated from averages of two sets of duplicate assays. The protein concentration of cell-free extracts was determined by comparison of optical density (OD_{575}) of duplicate samples, of 100 µl sample in 4.9 ml of Bradford Reagent, to a standard concentration curve of Bovine Serum Albumin (BSA) reacted with Bradford Reagent.

2.10.2 Catechol 2,3-Dioxygenase Assay

Catechol 2,3-dioxygenase cleaves the substrate catechol in the *meta* position to produce a yellow coloured product, 2-hydroxymuconic semialdehyde. The production of 2-hydroxymuconic semialdehyde can be measured spectrophotometrically at 375 nm. Assays were prepared by mixing 70 µl of cell-free extract with 700 µl of substrate (50 mM phosphate buffer containing 10% acetone and 10 mM catechol). The solution was assayed continuously at 375 nm for 180 seconds.

2.10.3 Catechol 1,2-Dioxygenase Assay

Catechol 1,2-dioxygenase catalyses the conversion of catechol to *cis, cis*-muconate via ring cleavage of catechol in the *ortho* position. Catechol 1,2-dioxygenase activity was assayed at 260 nm as described for catechol 2,3-dioxygenase. Catechol 2,3-dioxygenase was inactivated by the addition of 0.01% hydrogen peroxide (Hollender *et al*, 1990).

2.10.4 Benzyl Alcohol Dehydrogenase Assay

Benzyl alcohol dehydrogenase catalyses the conversion of benzyl alcohol to benzaldehyde. The assay for benzyl alcohol dehydrogenase was done following the method of Worsley and Williams (1975) except the assay buffer was exchanged for the buffer used in the previous assays (section 2.10.2 and 2.10.3). Assays were performed by mixing 70 µl of cell-free extract with 700 µl of substrate (50 mM phosphate buffer containing 10% acetone, and 7.5 µM NAD^+ (Boehringer Mannheim), and 8 µM benzyl alcohol (BDH)). The solution was assayed continuously at 340 nm for 360 seconds.

2.10.5 Benzaldehyde Dehydrogenase Assay

Benzaldehyde dehydrogenase catalyses the conversion of benzaldehyde to benzoate. This enzyme was assayed at 340 nm as described in 2.10.4 except 1.2 µM of benzaldehyde (BDH) was used in place of benzyl alcohol.

Chapter 3. Results

3.1 Antarctic Isolate Identification

All Antarctic isolates studied were gram negative, motile rods, 1-2 μm long and 1 μm wide and displayed the characteristics of the genus *Pseudomonas*. Bacterial colonies were large, mucoid, and creamy yellow in colour. All isolates tested were aerobic, oxidase positive, used arginine as a carbon source, and did not produce indole. Isolates 8/46, 7/163, and ant5 were identified as *Pseudomonas putida* and ant9 was identified as *Pseudomonas fluorescens*. Isolates 7/22, ant5, and ant9 were identified as *Pseudomonas* by 16S rRNA sequence analysis (Aislabie, J. personal communication).

3.2 Isolate Growth Characteristics

3.2.1 Solid Media

All naphthalene degrading isolates (Table 3.1) grew on BHA supplemented with naphthalene as a vapour at both 6°C and 15°C, except isolates 8/47 and 7/38 could not grow at 6°C. None of the isolates could grow on carbon-free BHA controls at 6°C and 15°C.

Table 3.1. Growth of naphthalene degrading isolates at 6°C and 15°C on BHA with naphthalene vapour supplied as the sole carbon source.

Isolate	6°C	15°C
8/47	-	+
7/157	+	+
7/156	+	+
7/38	-	+
ant5	+	+
ant9	+	+

All toluene degrading isolates (Table 3.2) were able to grow on BHA amended with 5 mM *meta*-toluate and BHA supplemented with 100 µl toluene as a vapour at 6°C and 15°C with the exception of 8/43 and 8/51, which did not grow on toluene at either temperature. None of the isolates grew on carbon-free BHA controls at 6°C and 15°C.

Table 3.2. Growth of toluene degrading isolates at 6°C and 15°C on BHA with toluene vapour or 5 mM *meta*-toluate supplied as a sole carbon source.

Isolate	*meta*-toluate		Toluene	
	6°C	15°C	6°C	15°C
8/46	+	+	+	+
8/43	+	+	-	-
8/51	+	+	-	-
7/163	+	+	+	+
7/167	+	+	+	+
7/22	+	+	+	+

3.2.2 Liquid Media

The toluene degrading isolates were able to grow in BH broth amended with 5 mM *meta*-toluate at 6°C (Figure 3.1) and 15°C (Figure 3.2). None of the toluene-degrading isolates could grow on BH supplemented with toluene vapour at 6°C (Figure 3.1) and 15°C (Figure 3.2). Controls revealed that none of the isolates were able to grow in liquid media (BH) without the addition of *meta*-toluate as a sole carbon source.

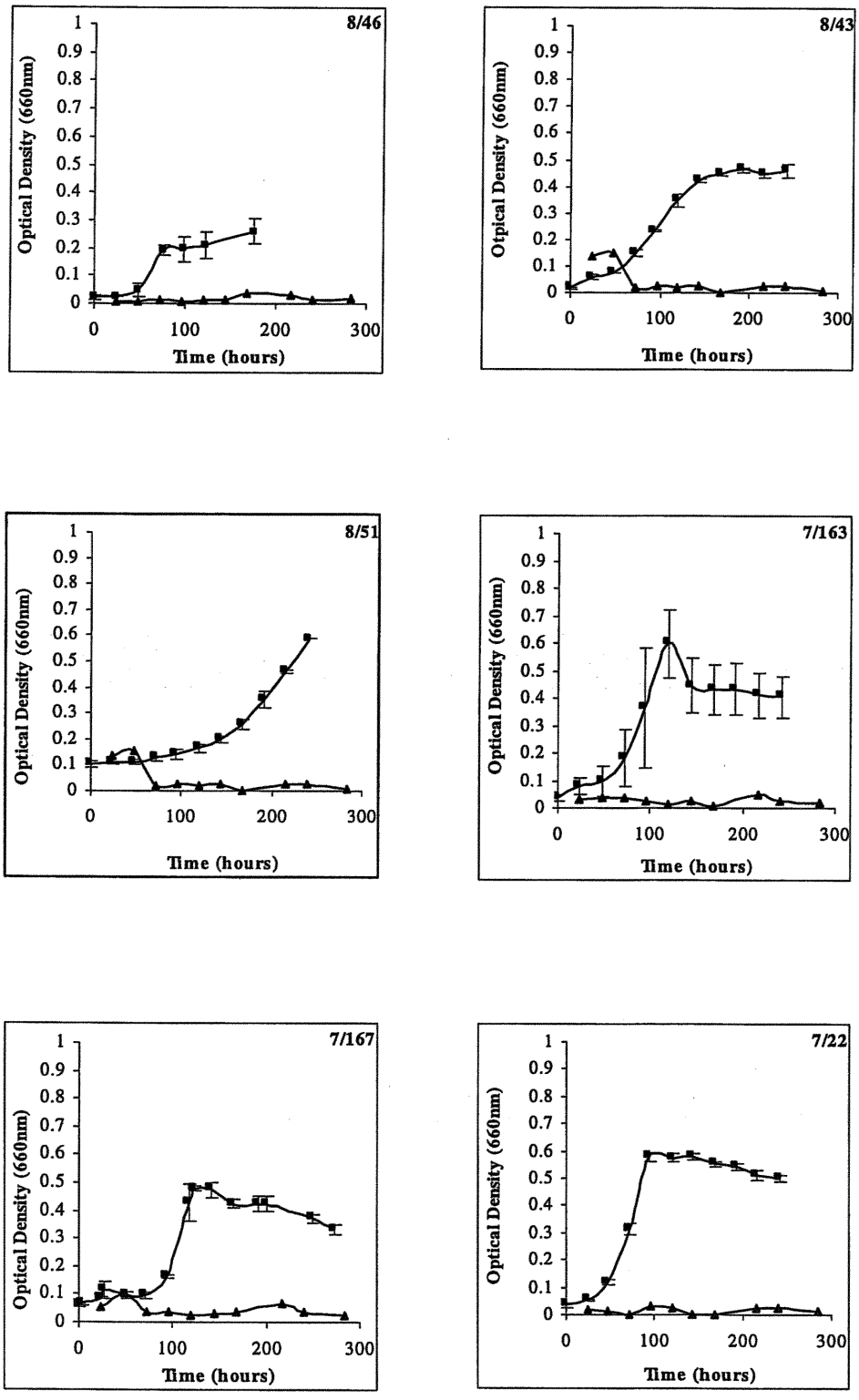


Figure 3.1. Growth of toluene degrading isolates at 6°C on toluene vapour and *meta*-toluate in liquid media. (Number of replicates =2. Error bars indicate ± standard deviation. ■ = *meta*-toluate ▲ = toluene)

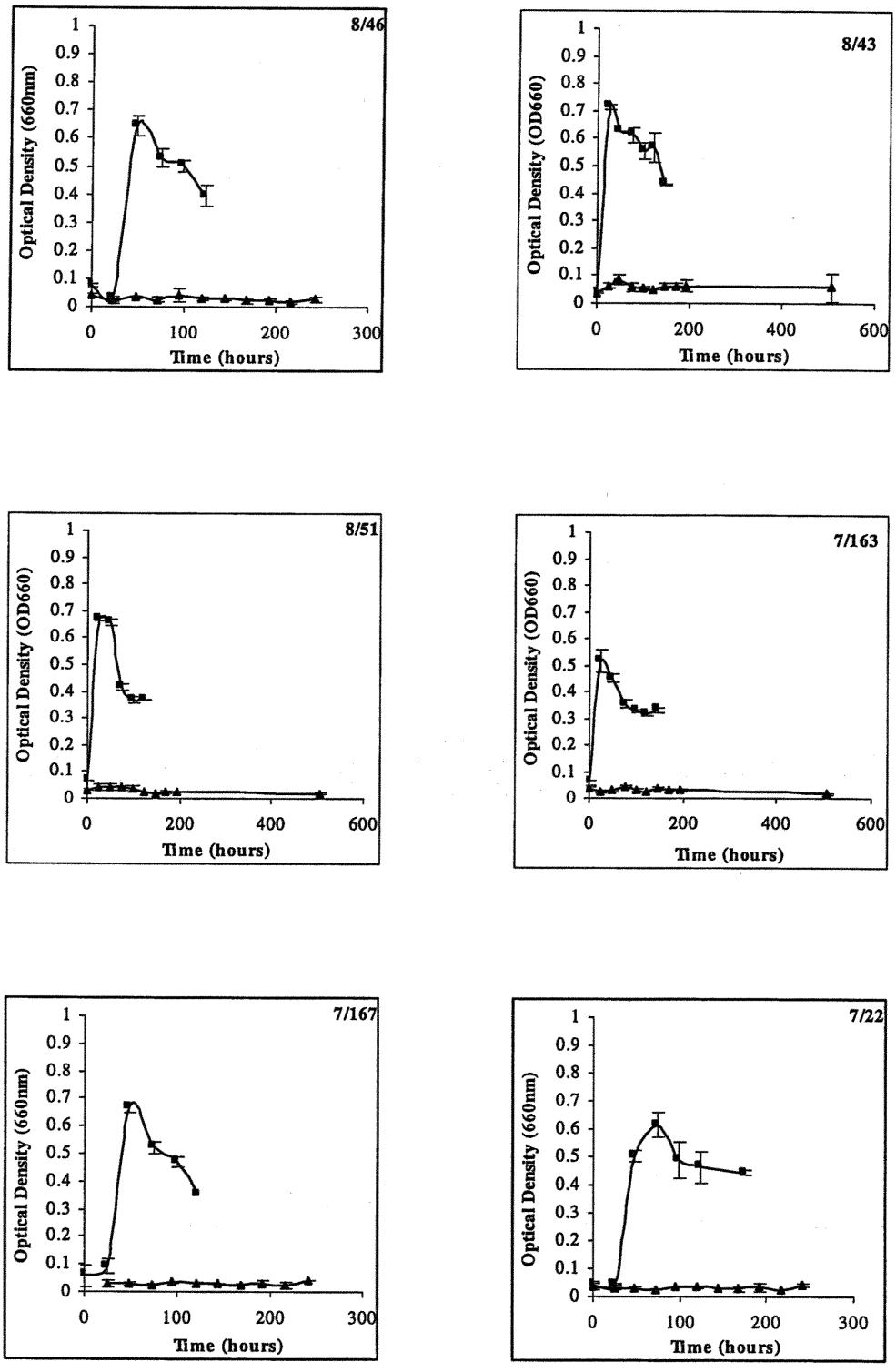


Figure 3.2. Growth of toluene degrading isolates at 15°C on toluene vapour and *meta*-toluate in liquid media. (Number of replicates =2. Error bars indicate ± standard deviation ■ = *meta*-toluate ▲ = toluene)

3.3 Effect of Temperature on Growth of Isolates 7/167 and 8/46 *meta*-toluate

Two toluene degrading Antarctic isolates, 7/167 and 8/46, were selected to determine their optimum growth temperature and the effect of temperature on the length of the lag phase of growth and cell density.

Isolate 7/167 grew on *meta*-toluate over a temperature range of 6°C to 30°C (Figure 3.3). Growth at 30°C was limited with a long lag phase of approximately 80 hours or three days. Growth at 6°C also showed a long lag phase of around three days. Incubation temperatures 20°C and 25°C showed the shortest lag phase, approximately 10 hours, and growth at 25°C reached a higher optical density than 20°C.

Isolate 8/46 grew on *meta*-toluate over a temperature range of 6°C to 30°C (Figure 3.4). In comparison to isolate7/167, growth of 8/46 at 30°C was high with a shorter lag phase of 24 hours. Isolate 8/46 grown at 6°C and 10°C showed limited growth and the lag phases at these temperatures extended out to 72 hours. When grown at 15°C isolate 8/46 reached the highest optical density (OD_{660}). Isolate growth at 20°C showed the shortest lag phase, approximately 5 hours, overall and the second highest optical density.

Isolates 7/167 and 8/46 were unable to grow in the carbon-free control BH flasks at any temperature tested.

Of the 30 individual toluene-degrading bacterial colonies (Section 2.5) subcultured from BHA-mtol to BHA supplemented with toluene vapour, and grown at 15°C, all were able to grow on toluene vapour as a sole source of carbon.

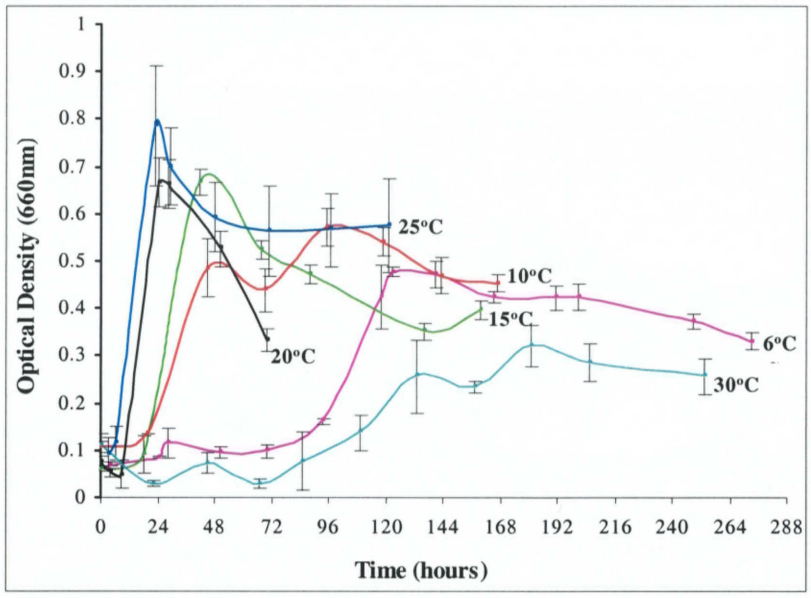


Figure 3.3. Growth of Antarctic isolate 7/167 on *meta*-toluate in liquid media at various temperatures. (Number of replicates=3. Error bars indicate ± standard)

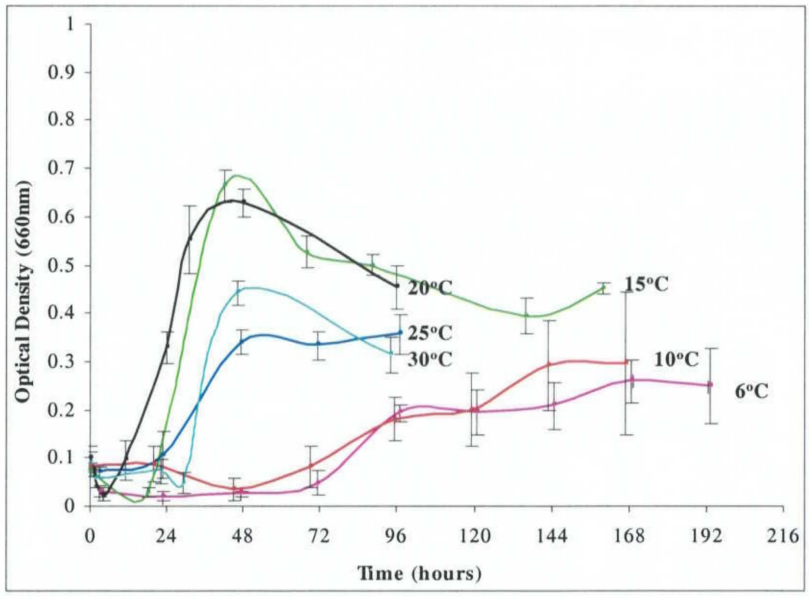


Figure 3.4. Growth of Antarctic isolate 8/46 on *meta*-toluate in liquid media at various temperatures. (Number of replicates =3. Error bars indicate ± standard deviation.)

Optimum growth rates were calculated for isolate 7/167 and 8/46 at each growth temperature tested. The optimum growth temperature for isolate 7/167 on *meta*-toluate is between 20°C and 25°C. The optimum growth temperature for isolate 8/46 on *meta*-toluate is 15°C (Figure 3.5).

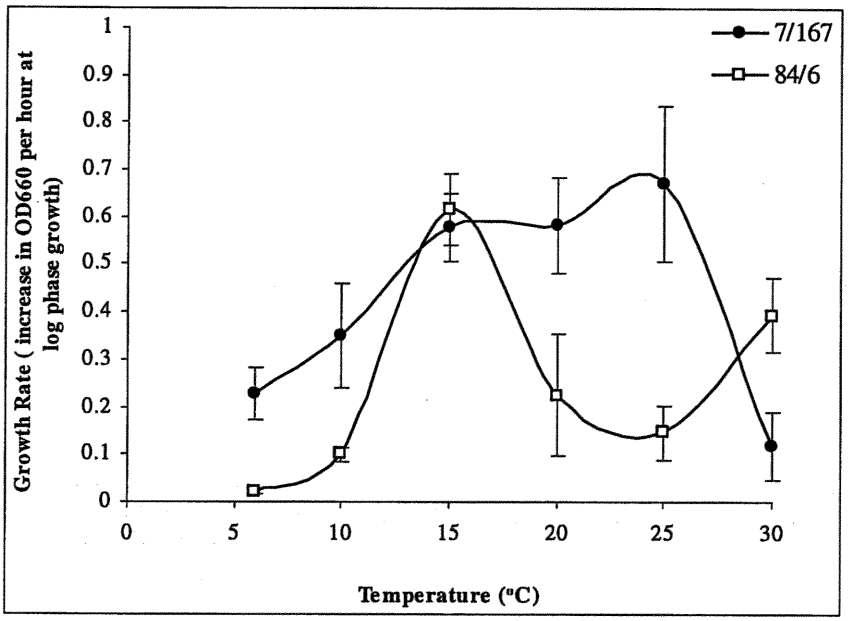


Figure 3.5. Maximum Growth Rates of Antarctic Isolates 7/167 and 8/46 on *meta*-toluate in liquid media at various temperatures. (Number of replicates=3. Error bars indicate ± standard deviation)

Sustained growth of isolate 7/167, on BH supplemented with 5 mM *meta*-toluate, at 6°C, 15°C, and 25°C and isolate 8/46 on *meta*-toluate at 6°C, 15°C, 25°C, and 30°C was assessed.

Isolate 7/167 was able to sustain growth on *meta*-toluate at 6°C, 15°C and 25°C (Figure 3.6) but could not sustain growth at 30°C. Growth of 7/167 on *meta*-toluate reached a higher optical density (OD_{660}) when grown at 25°C than when grown at 15°C and 6°C, optical densities of 0.8, 0.7, and 0.5, respectively. Cultures of 7/167 growing at 6°C were the only ones to show a slight lag phase of growth.

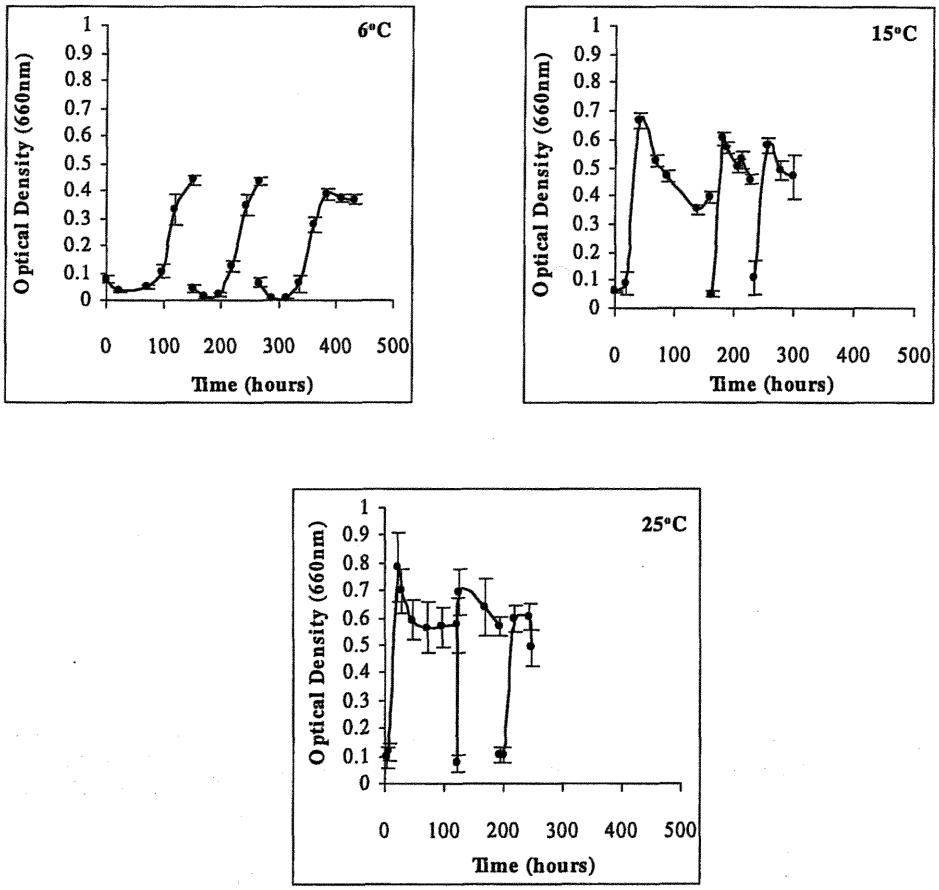


Figure 3.6. Sustained growth of Antarctic isolate 7/167 on *meta*-toluate in liquid media.

(Number of replicates=3 Error bars indicate ± standard deviation).

Isolate 8/46 was able to sustain growth on *meta*-toluate at 6°C, 15°C, 25°C, and 30°C (Figure 3.7). Isolate 8/46 growing at 15°C reached the highest optical density (OD_{660}) of the temperatures tested. Maximum growth densities (OD_{660}) at 6°C, 15°C, 25°C, and 30°C, were 0.25, 0.8, 0.7, and 0.6, respectively. Isolate 8/46 cultures grown at 6°C exhibited a long lag growth phase compared to growth on other temperatures tested.

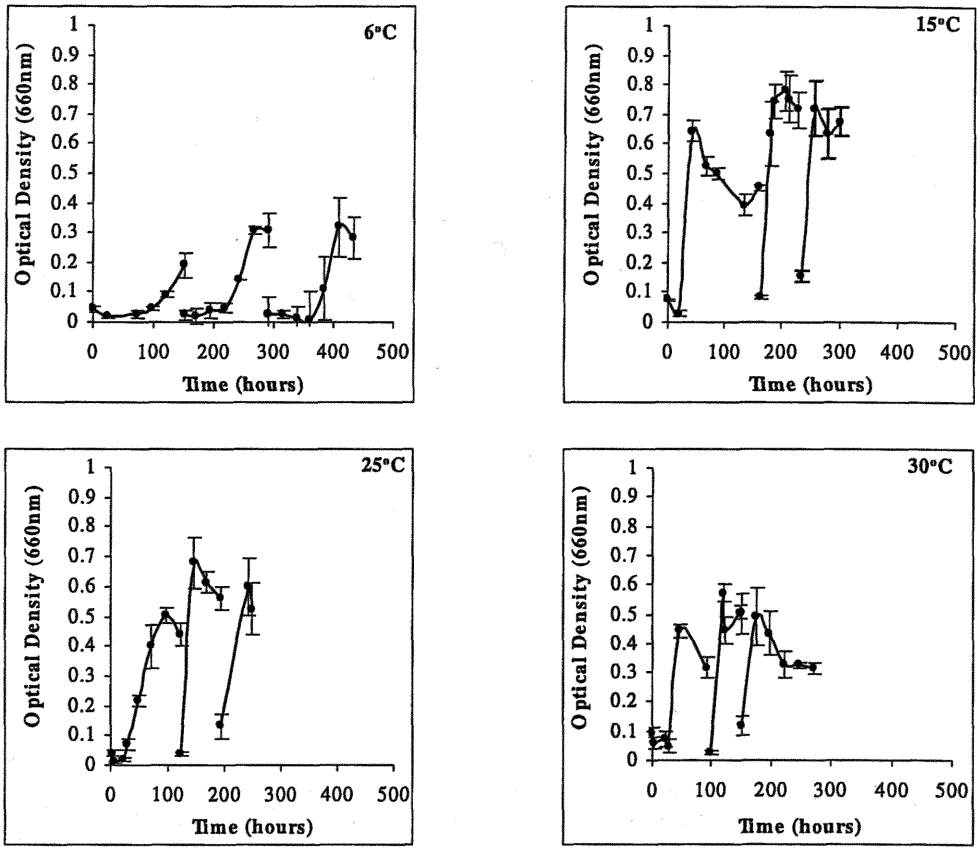


Figure 3.7. Sustained growth of Antarctic isolate 8/46 on *meta*-toluate in liquid media.

(Number of replicates=3 Error bars indicate ± standard deviation)

Both isolate 7/167 and 8/46 retained the ability to grow on BHA-mtol, BHA supplemented toluene vapour at 6°C, 10°C, 15°C, 20°C, 25°C, and 30°C after sustained growth at temperatures tested. Neither isolate could grow on carbon-free BHA at 6°C, 10°C, 15°C, 20°C, 25°C, and 30°C.

3.4 Plasmids Isolated and Restriction Digests of Plasmid DNA.

Plasmids were isolated by the Farrell plasmid isolation method (1980) from a number of toluene and naphthalene degrading Antarctic isolates (Table 3.3 and Figure 3.8). In all cases the plasmid DNA was contaminated with chromosomal DNA and restriction digest fragments were seen as a smear with ultraviolet light when electrophoresed on an agarose gel treated with ethidium bromide, consequently there was no further investigation of the plasmid DNA using this procedure.

Table.3.3. Toluene and naphthalene degrading Antarctic Isolates that had plasmids isolated from them by the Farrell plasmid isolation method.

Toluene-degrading Antarctic Isolates	Naphthalene-degrading Antarctic Isolates
8/46	7/156
7/163	7/38
7/167	ant5
7/22	ant9

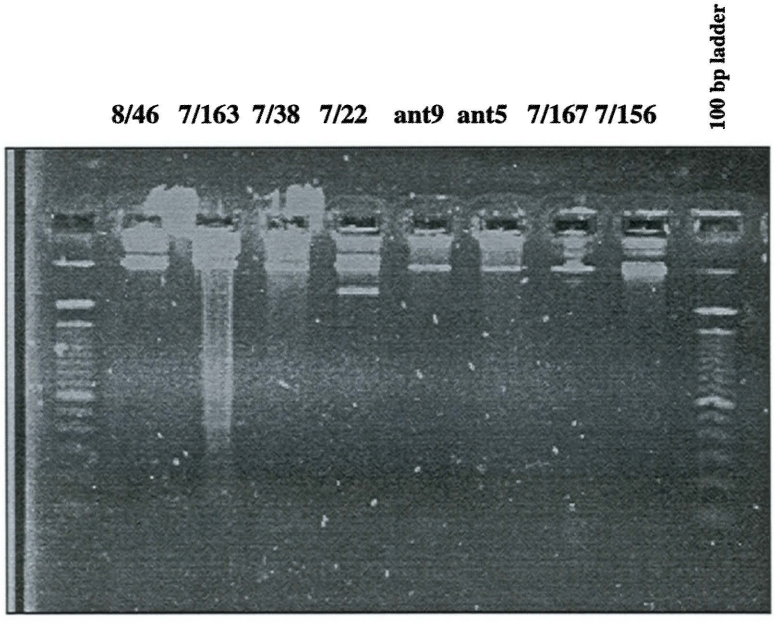


Figure 3.8. Plasmid DNA isolated from various toluene and naphthalene degrading Antarctic isolates.

Pure plasmid DNA was isolated from two toluene degrading isolates, 7/167 and 8/46, and the positive control isolate *P. putida* mt-2, using the Kieser Method (Kieser, 1984) (Figure 3.9). Isolate 7/167 shows an atypical plasmid DNA pattern with more than three bands observed, usually typical of open circular (OC) (one strand nicked), covalent closed circular (CCC) (no nicked strands), and linear (both strands nicked) plasmid DNA. Isolates 8/46 shows a more typical plasmid DNA pattern with the location of CCC most probably the fastest migrating band (Figure 3.9). SmaI, and XhoI digested plasmid DNA was electrophoresed and the fragment pattern observed for each plasmid shows that all three plasmids isolated are different in sequence and size (Figure 3.9).

Isolate 7/167 digested with SmaI results in the production of DNA fragments ranging in size from 2070 bp to 17760 bp. When digested with XhoI, 7/167 DNA was cleaved into fragments ranging from 1660 bp to 17960 bp (Table 3.4). Addition of restriction fragment sizes produced by XhoI indicates the plasmid isolated from 7/167 is 61 kb. The SmaI digestion fragment pattern of 7/167 plasmid DNA looks to be only partial as fragments total 35 kb.

Isolate 8/46 digested with SmaI results in the production of two DNA fragments of 5335 bp and 14240 bp. When digested with XhoI, 8/46 DNA was cleaved into fragments ranging from 3790 bp to 20950 bp (Table 3.6). Addition of restriction fragment sizes produced by XhoI indicates the plasmid isolated from 8/46 is 95 kb. The SmaI digestion fragment pattern of 8/46 plasmid DNA looks to be only partially digested, as fragments total 19.5 kb, signifying some of the fragment bands are not observable and may still be in the gel well or anomalous migration of fragments.

The positive control plasmid isolated from *P. putida* mt-2 was found to be 64 kb and 67 kb when digested with SmaI and XhoI, respectively (Table 3.4). The literature reports that the pWWO plasmid is 117 kb (Duggleby *et al,* 1977). This size discrepancy will be discussed in Section 4.3.

Table 3.4. Isolate 7/167, 8/46, and *P. putida* mt-2 plasmid DNA digestion fragment lengths produced by the restriction endonucleases SmaI and XhoI.

	Isolate 7/167		**Isolate 8/46**		***P. putida* mt-2 pWWO**	
	SmaI	**XhoI**	**SmaI**	**XhoI**	**SmaI**	**XhoI**
	17760	17960	14240	20950	15670	15670
	6230	13750	5335	15920	12125	13750
	4950	9560		12440	9560	10740
	4000	7670		11090	7670	6900
	2070	6230		9940	5650	6230
		3910		8945	3600	5130
		1660		6690	3300	3915
				5160	2820	2610
				3790	2610	1950
Plasmid Size (bases pairs)	**35020**	**60750**	**19575**	**94910**	**63600**	**66900**

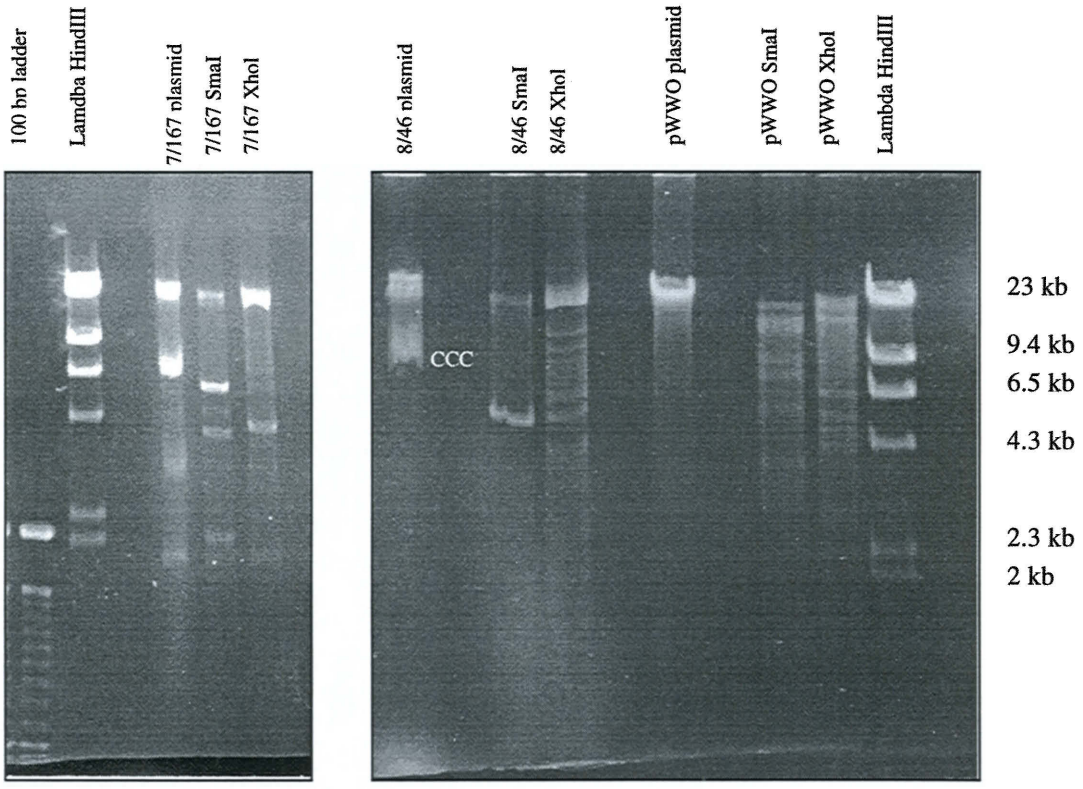


Figure 3.9. Plasmid DNA and restriction endonuclease digests of plasmid DNA isolated from Antarctic isolates 7/167 and 8/46 and the positive control isolate *P. putida* mt-2.

3.5 Polymerase Chain Reaction of Degradative Gene Sequences

The Polymerase Chain Reaction (PCR) was used to amplify regions of three genes from the TOL toluene degradative pathway. The genes for catechol 2,3-dioxygenase (*xylE*), benzyl alcohol dehydrogenase (*xylB*), and benzaldehyde dehydrogenase (*xylC*) were amplified from DNA isolated from Antarctic isolates 7/167 and 8/46 and from the positive control isolate *P. putida* mt-2.

Degradative gene regions were successfully amplified from *P. putida* mt-2 (Figure 3.10) for all three genes. Antarctic isolates 7/167 and 8/46 showed amplification of the *xylB* gene (Figure 3.10) and 7/167 amplification of the *xylE* gene but neither isolate had amplification of the *xylC* gene using the designed primers (Table 3.5) (Figure 3.10).

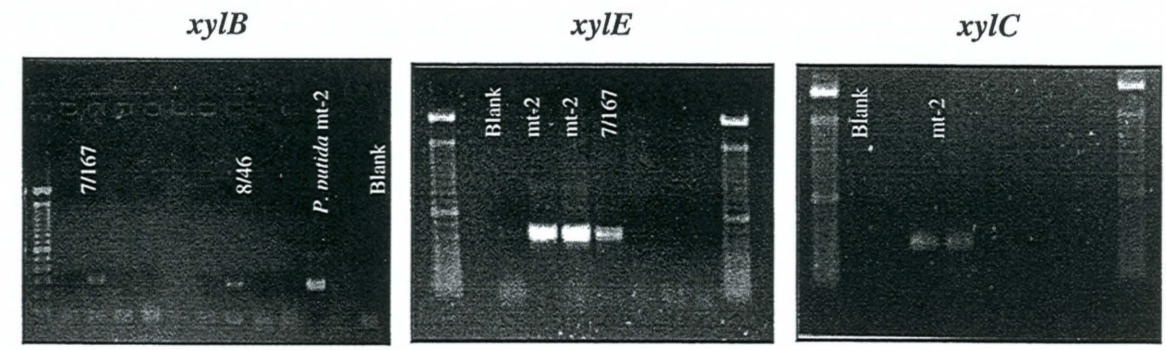


Figure 3.10. Polymerase Chain Reaction products from 7/167, 8/46, and *P. putida* mt-2 DNA when amplified with primers for benzyl alcohol dehydrogenase (*xylB*), catechol 2,3-dioxygenase (*xylE*), and benzaldehyde dehydrogenase (*xylC*).

Table 3 5. Toluene degradative gene regions amplified by Polymerase Chain Reaction.

Isolate	Catechol 2,3-dioxygenase (*xylE*)	Benzyl alcohol dehydrogenase (*xylB*)	Benzaldehyde dehydrogenase (*xylC*)
P. putida mt-2	+	+	+
7/167	+	+	-
8/46	-	+	-

The benzyl alcohol dehydrogenase gene (*xylB*) PCR products generated from the three isolates were the expected size, 277 bp. These three PCR products were sequenced (Appendix D) and the nucleotide sequences were aligned to the DNA sequence of the benzyl alcohol dehydrogenase (*xylB*) gene from *P. putida*-mt-2 using Blast Search (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-newblast) (Table 3.6).

Table 3.6. Percentage homology of Antarctic isolates, 7/167, and 8/46, benzyl alcohol dehydrogenase (*xylB*) PCR product to *P. putida* mt-2 benzyl alcohol dehydrogenase (*xylB*) gene.

Isolate	Length of DNA Sequence Aligned (base pairs)	% Homology to *P. putida* mt-2 benzyl alcohol dehydrogenase (*xylB*) gene
P. putida mt-2	240	99%
7/167	224	100%
8/46	86	92%

3.6 Degradative Enzyme Assays

Cell-free extracts from Antarctic isolates 7/167 and 8/46 were prepared from early and late log phase culture growth on *meta*-toluate. Cell-free extracts were assayed for the presence, as detected by activity, of four enzymes in the TOL toluene degradative pathway. The enzymes assayed were catechol 2, 3-dioxygenase, catechol 1, 2-dioxygenase, benzyl alcohol dehydrogenase, and benzaldehyde dehydrogenase (Figure 1.1). The relative specific activity of enzymes assayed is expressed as µmole product produced per minute per milligram protein in the cell-free crude extract for 7/167 and 8/46 (Table 3.7, 3.8, and 3.9). Assays of cell-free extracts of *P. putida* mt-2, at mid log phase of growth, showed the following relative specific activities; catechol 2,3-dioxygenase 0.233 µmol/min/mg total protein, benzyl alcohol dehydrogenase 0.0059 µmol/mimg total protein, benzaldehyde dehydrogenase 0.0043 µmol/min/mg total protein. Enzyme activity is also expressed as µmole product produced per minute per cell (Table 3.7, 3.8, 3.9)

3.6.1 Catechol 2,3-Dioxygenase

Catechol 2,3-dioxygenase catalyses the conversion of catechol to 2-hydroxymuconic semialdehyde. Cell-free extracts from Antarctic isolates 7/167 and 8/46 both had catechol 2,3-dioxygenase activity when assayed with catechol as a substrate (Table 3.7 and Figure 3.11). Enzyme activity was also observed by the visible change in substrate colour from clear (catechol) to yellow (2-hydroxymuconic semialdehyde). Catechol 2,3-dioxygenase activity per cell of isolate 7/167 was similar at early log phase growth (28 hours) and late log phase culture growth (50 hours) and enzyme activity was higher per cell than activity of isolate 8/46. Catechol 2,3-dioxygenase activity per cell of isolate 8/46 was higher at early log phase growth (28 hours) than late log phase growth (50 hours). Relative specific activity of this enzyme follows the same trend as the per cell enzyme activity for each isolate at each growth time. At early log phase 7/167 has a similar relative specific catechol 2,3-dioxygenase activity to *P. putida* mt-2.

Table 3.7. Catechol 2,3-dioxygenase activity of Antarctic isolates, 7/167 and 8/46, grown on *meta*-toluate.

Antarctic Isolate	Enzyme Activity 28hr Growth (early log phase)	Enzyme Activity 50hr Growth (late log phase)
7/167	2.94×10^{-14} $\pm0.38\times10^{-14}$	2.84×10^{-14} $\pm1.13\times10^{-14}$
	[^{rsa}0.238±0.031]	[^{rsa}0.16±0.011]
8/46	1.48×10^{-14} $\pm0.61\times10^{-14}$	0.92×10^{-14} $\pm0.12\times10^{-14}$
	[^{rsa}0.138±0.057]	[^{rsa}0.075±0.010]

Number of replicates=4. Average enzyme activity µmol/min/cell number ± standard deviation.

Relative Specific Activity (rsa)= µmol/min/mg total protein ± standard deviation

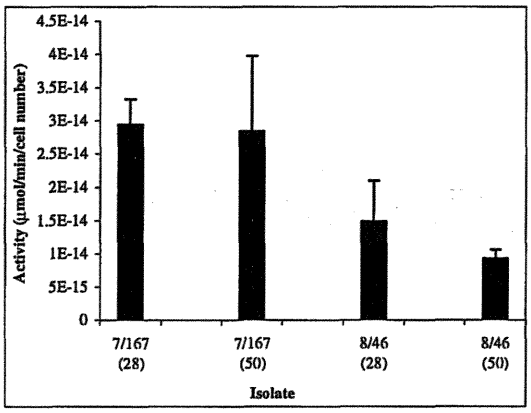


Figure 3.11. Catechol 2,3-dioxygenase activity of Antarctic isolates 7/167 and 8/46 grown on *meta*-toluate. (28 = signifies assay of sample after growth of 28 hours, early log phase growth, 50 = signifies assay of sample after growth of 50 hours late log phase growth. Number of replicates=4. Error bars = ± standard deviation).

3.6.2 Catechol 1,2-Dioxygenase

No activity was detected for catechol 1,2-dioxygenase for either of the isolates after 28 hours or 50 hours growth in BH supplemented with *meta*-toluate.

3.6.3 Benzyl Alcohol Dehydrogenase

Benzyl alcohol dehydrogenase catalyses the conversion of benzyl alcohol to benzaldehyde. Assay results indicate the presence of benzaldehyde in cell-free

extracts of Antarctic isolates 7/167 and 8/46. Benzyl alcohol dehydrogenase activity was observed in both isolates at both early and late log phase of culture growth (Table 3.8 and Figure 3.12). Isolate 7/167 showed similar benzyl alcohol dehydrogenase activity per cell at early and late log phase culture growth, but isolate 8/46 showed higher benzyl alcohol dehydrogenase activity per cell at early log phase growth (28 hours growth) than late log phase growth (50 hours). The relative specific activity of benzyl alcohol dehydrogenase in cell-free extracts of 7/167 and 8/46 is lower than activity of this enzyme in cell-free extracts of *P. putida* mt-2.

Table 3.8. Benzyl alcohol dehydrogenase activity of Antarctic isolates, 7/167 and 8/46, grown on *meta*-toluate

Antarctic Isolate	**Enzyme Activity 28hr Growth (early log phase)**	**Enzyme Activity 50hr Growth (late log phase)**
7/167	2.18×10^{-16} $\pm0.19\times10^{-16}$	2.1×10^{-16} $\pm0.79\times10^{-16}$
	[^{rsa}0.0017±0.0019]	[^{rsa}0.0011±0.002]
8/46	3×10^{-16} $\pm0.53\times10^{-16}$	1.1×10^{-16} $\pm0.57\times10^{-16}$
	[^{rsa}0.0031±0.002]	[^{rsa}0.0008±0.002]

Number of replicates=4. Average enzyme activity µmol/min/cell number ± standard deviation.

Relative Specific Activity (rsa)= µmol/min/mg total protein ± standard deviation

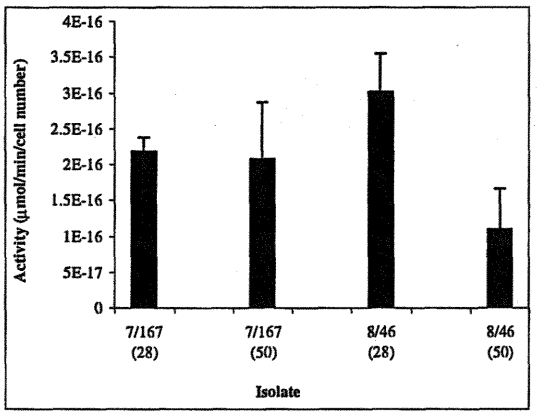


Figure 3.12. Benzyl alcohol dehydrogenase activity of Antarctic isolates 7/167 and 8/46 grown on *meta*-toluate. (28 = signifies assay of sample after growth of 28 hours, early log phase growth, 50 = signifies assay of sample after growth of 50 hours late log phase growth. Number of replicates=4. Error bars = ± standard deviation).

3.6.4 Benzaldehyde Dehydrogenase

Benzaldehyde dehydrogenase catalyses the conversion of benzaldehyde to benzoate. Assay results indicate the presence of benzaldehyde dehydrogenase in cell-free extracts of Antarctic isolates 7/167 and 8/46. Benzaldehyde dehydrogenase activity was observed in both isolates at both early and late log phase of culture growth (Table 3.9 and Figure 3.13). Isolates, 7/167 and 8/46, showed higher enzyme activity per cell (greater than three times activity) at early log phase of culture growth (28 hours growth) than late log phase culture growth (50 hours). Relative specific activity of benzaldehyde dehydrogenase in 7/167 and 8/46 cell-free extracts follows the trend as the per cell enzyme activity for each isolate at each growth time. At early log phase both 7/167 and 8/46 have higher relative specific activity of benzaldehyde dehydrogenase than *P. putida* mt-2.

Table 3.9. Benzaldehyde dehydrogenase activity of Antarctic isolates, 7/167 and 8/46, grown on *meta*-toluate.

Antarctic Isolate	**Enzyme Activity 28hr Growth (early log phase)**	**Enzyme Activity 50hr Growth (late log phase)**
7/167	7.1×10^{-16} $\pm0.69\times10^{-16}$	2.1×10^{-16} $\pm0.6\times10^{-16}$
	[^{rsa}0.0057±0.0005]	[^{rsa}0.0012±0.0002]
8/46	6.1×10^{-16} $\pm0.33\times10^{-16}$	1.5×10^{-16} $\pm0.24\times10^{-16}$
	[^{rsa}0.0052±0.0014]	[^{rsa}0.0012±0.0002]

Number of replicates =4. Average enzyme activity µmol/min/cell number ± standard deviation.

Relative Specific Activity (rsa)= µmol/min/mg total protein ± standard deviation

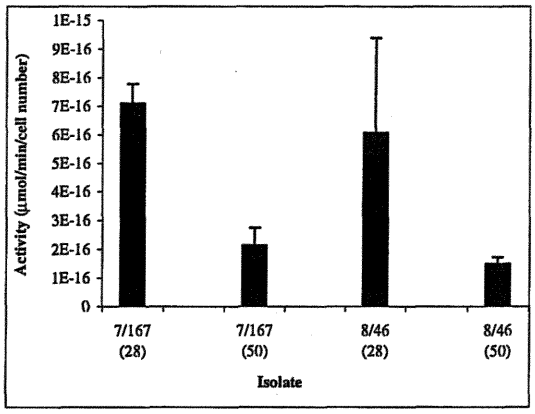


Figure 3.13. Benzaldehyde dehydrogenase activity of Antarctic isolates 7/167 and 8/46 grown on *meta*-toluate. (28 = signifies assay of sample after growth of 28 hours, early log phase growth, 50 = signifies assay of sample after growth of 50 hours late log phase growth. Number of replicates=4. Error bars = ± standard deviation).

Chapter 4
Discussion

4.1 Hydrocarbon Degrading Antarctic Pseudomonads

Indigenous hydrocarbon degrading Antarctic bacteria could be utilised to develop a bioremediation programme to clean up JP8 jet fuel contaminated sites at Scott Base, Antarctica. To assess the potential of Antarctic hydrocarbon degraders to degrade components of JP8 jet fuel, in temperatures similar to Antarctic soils, the effect of temperature on isolate growth was assessed. The optimum growth temperatures and temperature growth ranges for two toluene degrading isolates were determined in order to clarify whether Antarctic isolates do grow on hydrocarbons as a sole carbon source at low temperatures and thus have the potential to be used to develop a bioremediation programme in Antarctica.

The Antarctic isolates used in this study are motile, aerobic, gram negative rods and are classified as presumptive *Pseudomonas* species. Isolates 7/22, ant5, and ant9 have been identified as *Pseudomonas* by 16S rRNA sequence analysis (Aislabie, J, personal communication). Identification of indigenous hydrocarbon degrading Antarctic bacteria, used in this study, by API 20 NE test strips revealed all isolates tested belong to the genus *Pseuodomonas* and are further identified as *P. putida* or *P. fluorescens.* Antarctic isolates need to be investigated further ie, by 16S RNA sequence analysis to identify them correctly at the species level.

4.2 Growth Characterisation of Antarctic Isolates

Indigenous toluene and naphthalene degrading Antarctic isolates were isolated from JP8 jet fuel contaminated soils at Scott Base, Antarctica. All naphthalene degrading isolates were able to grow on solid media supplemented with naphthalene as a sole carbon at 15°C but only four of the six isolates could grow at 6°C. All the toluene degraders were able to grow on solid and liquid minimal media supplemented with 5 mM *meta*-toluate at 6°C and 15°C whereas only four of these six isolates could grow

on solid media supplemented toluene and none could grow on toluene in liquid media. The lack of isolate growth on toluene in liquid media suggests the concentration of toluene vapour supplied to the cultures was inhibitory or lethal to cell growth. Vercellone-Smith and Herson (1997) found that 1% toluene in a total volume of 5 ml of hexadecane (4 mg toluene/litre) supported cell growth whereas 25% toluene (130 mg/litre) is inhibitory to cell growth and 100% (267 mg/litre) is lethal to cells. The growth on toluene in liquid media experiments in this thesis used 100% toluene and thus subjected the cultures to lethal levels of toluene vapour. This explains why no cell growth was seen in these experiments. As stated when grown on solid media supplied with toluene vapour as a sole carbon source four of the six toluene degrading isolates grew at 6°C and 15°C. This result indicates that these toluene degrading isolates have the metabolic capability to utilise toluene as a carbon source and if supplied with the correct concentration of toluene vapour in liquid media it is expected that these isolates would be able to grow on toluene. To determine if the toluene degrading isolates can metabolise toluene as a sole carbon source when grown in liquid media, the growth experiments should be repeated using a lower concentration of toluene. Muller *at el*, (1990) used Tween 80 to increase the polycyclic aromatic hydrocarbon (PAH) concentration in aqueous solution so the compound is more available to degradation by microbes. This method could be used to provide the toluene degraders with a soluble solution of toluene in liquid media as opposed to toluene vapour. This method would also be conducive to measuring the concomitant decrease in toluene concentration, by high pressure liquid chromatography, and increase in cell biomass, spectrophotometrically thus confirming microbial metabolism of toluene.

Two toluene degrading isolates, 7/167 and 8/46, were selected for further in-depth growth characterisation. Isolate 7/167 was isolated from the site of a JP8 jet fuel leak near the Scott Base kitchen and isolate 8/46 for the site of a leaking pipeline carrying JP8 jet fuel to Williams Field. These two isolates were selected in order to assess any variability in toluene degrading isolates at these contaminated sites. Isolates were tested for growth on *meta*-toluate as a sole carbon source, due to their lack of growth on toluene vapour. Both isolates were able to grow over a range of temperatures from 6°C to 25°C. Isolate 7/167 was unable to sustain growth at 30°C and 8/46 could

sustain growth at 30°C but could not grow at 35°C. When transferred from *meta*-toluate growth flasks, at each temperature, to solid media supplemented with toluene vapour all isolates were able to grow. It can be concluded that sustained growth on *meta*-toluate as a sole carbon source does not causes the isolates, 7/167 and 8/46, to lose their ability to metabolise toluene as a carbon source.

Determination of maximum growth rates showed that 7/167 has an optimum temperature of 20–25°C and 8/46 has an optimum of 15°C. Based on definitions by Morita (1975) and Gow and Mills (1984) psychrophiles have an upper growth limit of 20°C and a minimal growth limit of 0°C or below, whereas psychrotrophs grow well at 20°C and are able to grow up to 30–35°C, but grow poorly at 5°C. Following these definitions the growth characteristics of these Antarctic toluene degrading isolates indicate that they are psychrotrophic not psychrophilic. It is noteworthy that some toluene degrading *Pseudomonas* species are able to grow on toluene to a maximum temperature of 30°C but are able to grow up to 37°C when grown on nutrient rich media (Chablain *et al*, 1997). Testing the Antarctic isolates for maximum growth temperature when growing on nutrient rich media would clarify the growth range of these isolates and determine if they are indeed psychrotrophs or grow at temperatures typical of both psychrotrophs and mesophiles.

Psychrophilic bacteria have been isolated from Antarctic sea ice and waters. Gosink *et al*, (1997), isolated a strain of *Octadecabacter* from Antarctic sea-ice and water that has a narrow temperature growth range of 4°C–10°C. However the majority of bacteria in Antarctic terrestrial environments are psychrotrophic not psychrophilic (Ellis-Evans, 1985). This is due to the variation of temperatures occurring in Antarctic soil environments as opposed to the stable temperatures psychrophiles inhabit ie, sea-ice and water (Ellis-Evans, 1985). The Scott Base soil temperatures fluctuate (Campbell and MacCulloch, 1997), and thus would be more conducive to the survival of psychrotrophs as they have a more flexible growth temperature range than do psychrophiles.

Mean annual air temperatures at Scott Base range from -4°C in January to -30°C in July and August, although temperatures may rise above these mean monthly

temperatures due to the proximity of Scott Base to the coast (Campbell and Claridge, 1987). In the summer months the surface soil temperature remains warmer than the overlying air due to 24-hour insolation (Campbell and MacCulloch, 1997). Campbell and MacCulloch (1997) found that the surface soil temperature at Scott Base, on a clear-sky day in the 1994 summer season ranged from between +17.8°C to -4.7°C (22°C range) and peaked at a +17.8°C for 30 minutes. They also found that at 5 cm soil depth the temperature range was 7.5°C. The bacteria used in this study were isolated from soil (0–5 cm depth) at Scott Base. As previously stated the optimum growth rate temperature for the two Antarctic isolates, 7/167 and 8/46, was 20–25°C and 15°C, respectively, so on some clear-sky days in the Antarctic summer these isolates could be growing at near optimal rate for 30 or more minutes. The temperatures that support maximum growth rate of toluene degrading Antarctic isolates *ex situ* are within 2–3°C of the maximum soil temperatures found at Scott Base, and this could indicate a temperature adaptation by these isolates. A further indication of adaption of Antarctic toluene degraders to temperature is the extended lag phase they exhibit when growing on *meta*-toluate at 6°C.

When grown at 6°C on *meta*-toluate as a sole carbon source both isolates, 7/167 and 8/46, showed a long lag phase of growth (~72 hours) compared to growth at higher temperatures ie, 20°C. This extended lag phase could be caused by (1) the induction and expression of cold shock proteins (csps), cold acclimation proteins (caps), and the enzymes for toluene degradation and/or (2) a decreased in enzyme activity at low temperatures. Vishniac (1993) suggests the most important aspects of temperature for Antarctic microbial life are the lengths of the growing season and the frequency of the freeze-thaw cycles. Freeze-thaw cycles occur daily on the surface soil at Scott Base, though fewer cycles occur at a 3 cm soil depth (Campbell and Claridge, 1987). The possible presence of cold shock and cold acclimation proteins may protect these microbes from the effects of daily freeze-thaw. Ray *et al*, (1994), found that a psychrotrophic Antarctic *Pseudomonas fluorescens* strain expressed a cold shock gene, homologous to the cold shock gene *cspA* of *Escherichia coli*, at a 10 fold higher level when grown at 4°C compared to 22°C. They also found that of the eight Antarctic *P. fluorescens* strains tested six contained *cspA* DNA sequences in their genome and that

the *cspA* gene appears to be a transcription factor for the expression of cold-inducible genes or an antifreeze protein.

The biodegradative *hut* operon (histidine utilisation operon) of an Antarctic *Pseudomonas* species has increased expression at low temperatures but the enzymes expressed, histidase and urocanase, became less active as the temperature decreased (Kannan *et al,* 1998). The authors postulated that the isolate produces more of the degradative proteins at lower temperatures (4°C) to compensate for the loss of enzyme activity at this temperature.

P. putida Q5T, a transconjugant of the psychrotrophic bacterium *P. putida* Q5 containing the toluene-degrading TOL (pWWO) plasmid, expressed a greater number of cold acclimation proteins (caps) and cold shock proteins (csps) when grown at 5°C on *meta*-toluate, than when grown on *meta*-toluate at 25°C or salicylate at 5°C and 25°C (Gumley and Innis, 1996). When grown on salicylate at 5°C the transconjugant produced five caps, compared to nine caps when grown on *meta*-toluate. The increased protein expression by *P. putida* Q5T, compared to *P. putida* Q5 (does not contain the TOL plasmid), when grown on *meta*-toluate and at cold temperatures may indicate that expression of the inserted TOL plasmid may add addition stress due to the increased metabolic load (Gumley and Innis, 1996).

An increased metabolic load due to increased levels of cold shock proteins and cold acclimation proteins and degradative enzyme production could explain the reason for the long lag phase observed when toluene degrading Antarctic *Pseudomonas* species are grown at low temperatures (6°C) on *meta*-toluate. The findings of other authors and the long lag phase observed from these Antarctic bacteria growth experiments could suggest that these degradative Antarctic bacteria may have mechanisms for sustaining growth in cold, polluted Antarctic soils.

The fact that soil temperatures at Scott Base, Antarctica, during the summer months appear conducive to microbial growth and that Aislabie *et al*, (1998) found that numbers of culturable Antarctic hydrocarbon degrading bacteria were enhanced in oil contaminated soils compared to pristine soils suggests that hydrocarbon-degraders are

capable of growth, on components of oil, in Antarctic soils. Based on this information and the microbial growth temperature ranges found in this study, bioremediation would be a feasible option to clean up JP8 jet fuel contaminated sites at Scott Base, Antarctica. The Antarctic toluene degrading isolates studied can grow and sustain growth on *meta*-toluate, as a sole carbon source at temperatures similar to those that occur in soils at Scott Base, Antarctica, over the summer months. These isolates therefore have the potential to be used to develop a successful bioremediation program to clean up JP8 jet fuel contaminated sites at Scott Base, Antarctica.

In order to understand more about the toluene degradative mechanism used by the toluene degrading Antarctic isolates 7/167 and 8/46, their degradative genes and enzymes involved in toluene degradation were studied.

4.3 Biodegradative Plasmids in Hydrocarbon Degrading Antarctic isolates

Little is known about the mechanism of toluene degradation by Antarctic bacteria. The genes encoding the toluene degradative pathway have been found to be plasmid born or chromosomally encoded in toluene degrading bacteria isolated from other continents (Frantz and Chakrabarty, 1986; Kaphammer *et al*, 1990; Whited and Gibson, 1991; van der Meer *et al*, 1992; Williams and Sayers, 1994; Shields *et al*, 1995). The commonest route of toluene degradation by pseudomonads is encoded by the TOL group of plasmids and involves degradation of toluene through the *meta* cleavage pathway to acetaldehyde and pyruvate (Williams and Sayers, 1994). There is little in the literature about the presence of plasmids in Antarctic *Pseudomonas* species, although Shivaji *et al* (1994) isolated two *Pseudomonas* species from the Schirmacher Oasis, Antarctica, that contained plasmids of ~85, ~4, and ~4.3 kilobase pairs in size. The function of these plasmids was not discussed. Large size tends to be a feature of biodegradative plasmids and this study found that both toluene and naphthalene degrading Antarctic isolates harbour large plasmids.

This study isolated presumptive plasmids, using the Farrell plasmid isolation method, from a number of toluene and naphthalene degrading bacteria that had been isolated

from JP8 jet fuel contaminated soils at Scott Base, Antarctica (figure 3.8). The plasmid and linear chromosomal DNA was isolated and able to be separated by their migration in a salt gradient, but collection of bands of plasmid DNA proved difficult due to the large size of these plasmids, a feature of biodegradative plasmids in general. An upper band (chromosome) and a lower band (covalently close circle plasmid DNA) were seen in salt gradients performed on isolated Antarctic DNA. The observation of two bands in the gradient indicated that a plasmid was present in the isolate, as did the isolates' ability to metabolise toluene or naphthalene as a sole carbon source, as this metabolic ability is commonly plasmid encoded in *Pseudomonas* species (Frantz and Chakrabarty, 1986). These presumptive plasmids isolated were unable to be identified further, by restriction digest fragment pattern, due to chromosomal DNA contamination of the plasmid DNA. Digested chromosomal DNA tends to appear as a smear when electrophoresed on an agarose gel and obscures any plasmid fragments present. Of some interest was the visible presence of multiple (4) faint upper bands and one strong lower band from DNA isolated from isolate 7/163. This, combined with the atypical plasmid DNA pattern seen for plasmid DNA isolated from 7/167 (figure 3.9), using the Kieser plasmid isolation method, could indicate the presence of multiple plasmids, or multiple forms of the same plasmid, or multiple forms of multiple plasmids in these two toluene degrading isolates.

Pure plasmid DNA was isolated, using the Kieser plasmid isolation method, from the toluene degrading Antarctic isolates 7/167 and 8/46, and from the positive control *P. putida* mt-2, harbouring the pWWO TOL plasmid (Figure 3.9). Isolate 8/46 has a typical plasmid pattern, with the lower band being the covalently closed circle (CCC) form of the plasmid, and as stated 7/167 has an atypical plasmid pattern with 4 distinct bands of DNA observed that could indicate the presence of multiple plasmids. Duggleby *et al*, (1977), isolated two *Pseudomonas* strains each harbouring two different sized plasmids. Foght and Westlake (1988) isolated a PAH (polycyclic aromatic hydrocarbon) degrading *Pseudomonas* species that contained four plasmids ranging from 37 to 6×10^6 megadaltons. A NAH plasmid (naphthalene degradative plasmid) and a plasmid that hybridised to an *alkB* gene probe (alkane degrading plasmid) was found in a hydrocarbon degrading *Pseudomonas* species isolated from petroleum contaminated Arctic soil (Whyte *et al*, 1997). Farrell (1980) observed

multiple plasmids and multiple forms of plasmids (supercoiled, CCC, linear), in a naphthalene degrading *P. putida* strain, by electron microscopic examination of the isolates' DNA. To clarify the existence of multiple plasmids or multiple forms of plasmid(s) in Antarctic isolates 7/167 and 7/163 electron microscopic observation or gradient centrifugation of the plasmid(s) would be required.

DNA isolated from 7/167, 8/46, and pWWO plasmid DNA was digested with the restriction enzymes SmaI and XhoI. The pWWO plasmid of *P. putida* mt-2 is known to be 117 kb pairs in size (Duggleby *et al*, 1977). The results of SmaI and XhoI digestion of pWWO in this thesis show a total plasmid size of 64 kb and 67 kb, respectively, which is half of the size stated in the literature (figure 3.9). Duggleby *et al*, 1977, determined the size of the pWWO TOL plasmid by electron microscopic observation of the DNA, using ColEI and RP1 as internal size standards. The size was confirmed by densitometry trace of an EcoRI digest of the pWWO plasmid DNA.

A possible explanation for the difference in pWWO plasmid size between this thesis and the literature could be due to the presence of a transposable element on this plasmid. Frantz and Chakrabarty (1986) identified a 56 kb transposable element, containing the degradative pathway genes, on a TOL plasmid. This transposon was able to duplicate as indicated by the duplication of the plasmid encoded catechol 2,3-dioxygense gene in various bacterial strains (Chatfield and Williams, 1986). It is possible that the pWWO plasmid, of *P. putida* mt-2, used in this study has lost one of its original copies of this transposon, possibly to the chromosome, and is now 56 kb small (61 kb) than the original 117 kb pWWO plasmid. The sizes of pWWO plasmid found in this study, 64 and 67 kb, is relatively close to 61 kb. Another possible explanation of the size difference of pWWO is the reported evidence that a TOL plasmid in a *P. putida* or in a *P. aeruginosa* PAO host, grown on benzoate, dissociates, mediated by RecA dependent specified host recombination factors, to form two individual plasmids. One plasmid harboured the degradative genes and the other acted as a transfer plasmid (Farrell, 1980). It is possible that a transfer plasmid exited as part of the original pWWO plasmid but the isolates subsequent growth on benzoate has caused it to lose the transfer part of the original plasmid and consequently the size of the pWWO plasmid is reduced.

When Antarctic isolate, 7/167 and 8/46, DNA was digested with SmaI and compared XhoI digests the results suggest only partial digestion of DNA occurred using the SmaI enzyme. DNA from 8/46 and 7/167 produced only 2 and 5 fragments, respectively, when digested with SmaI. The top fragment in each case appears to be undigested plasmid DNA because these bands migrated to a similar distance on the gel as the undigested plasmid DNA. The DNA digests generated by the XhoI restriction enzyme produced more fragments, from 7/167 and 8/46 plasmid DNA, than SmaI digests although this DNA also appeared to be incompletely digested. Results of digestion of 7/167 DNA with XhoI shows this isolate has a fragment pattern, and fragment sizes similar to pWWO. The total size of the plasmid in Antarctic isolate 7/167 is 61 kb and this is within 3 kb of the size of pWWO (64 and 67 kb) according to the digest results in this thesis. The digest fragment pattern of 8/46 plasmid DNA with XhoI shows at least 10 DNA fragments generated although a number of these bands are faint (Figure 3.9). The total size of the plasmid in Antarctic isolate 8/46 is 95 kb. The plasmid isolated from 8/46 is different from the plasmid isolated from 7/167, as it is bigger in total size, generates a different number and sized DNA fragments when digested with both SmaI and XhoI. The difference in DNA fragment patterns between the two plasmids indicates that the DNA sequence of the two plasmids differs. The plasmid from 7/167 is similar to pWWO in DNA fragment sizes. Duggleby *et al* (1977) isolated large plasmids, ranging in size from 37 kb to 304 kb, from a number of toluene degrading bacterial strains and these digest results show that large plasmids, a common feature of biodegradative plasmids in general, are present in the two toluene degrading Antarctic isolates, 7/167 and 8/46, studied.

Both 7/167 and 8/46 plasmid DNA was not fully digested by either SmaI or XhoI. This is in contrast to both pWWO digests. All restriction digests were run at the same time, and under the same conditions. The lack of complete digestion of Antarctic DNA could indicate that the concentration of this DNA in the digest solution was too high for digestion under these conditions or that the Antarctic DNA was significantly physically different ie. coiled or covered in proteins and therefore not accessible for cleavage by restriction enzymes. Digestion of a series of different concentrations of Antarctic plasmid DNA would likely give a clearer restriction digest result and closer approximation of plasmid size in these two isolates.

4.4 Toluene Degradative Genes and Enzymes in Antarctic Isolates 7/167 and 8/46.

Polymerase Chain Reaction was used to assess the presence of three toluene degradative genes, *xylB*, *xylC*, and *xylE*, in Antarctic isolates 7/167 and 8/46. These *xyl* genes are commonly involved in toluene degradation by pseudomonads and are involved in the toluene degradative pathway encoded by the archetypal pWWO TOL plasmid of *P. putida* mt-2, and maybe involved in toluene degradation by the Antarctic toluene degraders. The pWWO plasmid from *P. putida* mt-2 was used as a positive control when assessing presence of degradative genes. Assays of four enzymes commonly involved in toluene degradation by pseudomonads, benzyl alcohol dehydrogenase, benzaldehyde dehydrogenase, catechol 2,3-dioxygenase, and catechol 1,2-dioxygenase, were performed on 7/167 and 8/46 to determine the presence enzymes involved in toluene degradation in these isolates and to determine the mechanism of toluene degradation by these isolates.

4.4.1 Toluene Degradative Genes

The degradation of toluene, *m*-xylene, *p*-xylene, and their corresponding acid and alcohol derivatives, through the *meta*-pathway, is encoded by the *xyl* genes on TOL plasmids and is the commonest route of toluene degradation by pseudomonads (Williams and Sayers, 1994). The three *xyl* genes selected for study are *xylB* (benzyl alcohol dehydrogenase), *xylC* (benzaldehyde dehydrogenase), and *xylE* (catechol 2,3-dioxygenase). These three genes are part of the TOL toluene degradation pathway encoded by the archetypal TOL plasmid, pWWO, and have been sequenced. DNA isolated from Antarctic isolates 7/167 and 8/46 was assessed for the presence of these three *xyl* degradative gene sequences

The *xylB* and *xylC* genes are encoded by the upper TOL toluene degrading pathway (Burlage *et al*, 1989). The *xylE* gene is coded on the lower (*meta*) pathway of the TOL plasmid. DNA isolated from 7/167, 8/46, and *P. putida* mt-2, by the Farrell plasmid isolation method, was used as a DNA template to amplify any DNA sequences homologous to *xylB*, *xylC*, and *xylE*. The DNA isolated by the Farrell method contained plasmid and chromosomal DNA and was used as a template for PCR of the

three degradative genes in order to ensure that chromosomal or plasmid encoded *xylB, xylC,* or *xylE* genes would be detected. The PCR protocols for amplification of *xylB, xylC,* and *xylE* genes were taken from Whyte *et al,* (1996). The primers for amplification of regions of the *xylB* and *xylC* degradative genes were designed from pWWO DNA sequence (www.ncbi.nlm.nih.gov/htbin-post/Entrez/nucleotide.html). The *xylB* primers should have produced a PCR product 277 bp in size. A PCR product of ~300 bp was generated from *P. putida* mt-2, 7/167, and 8/46 DNA (Figure 3.10). Sequence analysis of the *xylB* PCR products and comparison to the *P. putida* mt-2 sequence, from which the *xylB* primers were designed, revealed that the DNA amplified from 7/167 is 100% homologous to the *P. putida* mt-2 sequence. DNA from 8/46 is 92% homologous to the *P. putida* mt-2 sequence, although a smaller region of homology was found, 86 bp compared to 224 bp and 240 bp from 7/167 and *P. putida* mt-2 control, respectively (Table 3.8). DNA from *P. putida* mt-2, acting as a positive control, is 99% homologous to the *P. putida* mt-2 sequence. The 1% difference in DNA sequence is likely due to a nucleotide error generated by the DNA sequencing reaction.

The *xylC* primers should have produced a 496 bp PCR product from the pWWO plasmid isolated from *P. putida* mt-2. Instead a 300-400 bp product was generated from *P. putida* mt-2 DNA isolated by the Farrell and Kieser methods (Figure 3.10). This is likely due to non-specific primer binding to *P. putida* DNA. This experiment should be repeated using new primers. No PCR products were produced from 7/167 and 8/46 DNA using *xylC* primers. These results indicated the primers were not specific for 7/167 and 8/46 DNA and new primers should be designed and tested to clarify the presence of *xylC* in 7/167 and 8/46.

The *xylE* primers, taken from Whyte *et al,* (1996), generated the correct sized PCR product (~800bp) from DNA isolated from *P. putida* mt-2 by both the Farrell and Kieser plasmid isolation methods. Four PCR products from 7/167 DNA and two PCR products from 8/46 DNA were generated suing the *xylE* primers. None of the PCR products from 7/167 and 8/46 were ~800 bp in size. The *xylE* primers lacked specificity to 7/167 and 8/46 DNA, this could have been due to the present of chromosomal DNA in the sample or that the DNA sequences differ in Antarctic isolate

DNA compared to *P. putida* mt-2. Primer annealing temperature, Magnesium chloride, DNA, and primer concentration gradients, as well as number of PCR cycles were varied to maximise primer-binding specificity. In all cases multiple PCR products were generated from 7/167 and 8/46 DNA, with the exception of a 30 cycle PCR which generated no PCR products. None of the multiple PCR products were able to be individually resolved for DNA sequencing reactions. A new *xylE* reverse primer was designed from the *xylE* gene DNA sequence from the pWWO plasmid in *P. putida* mt-2 (http://www.ncbi.nlm.nih.gov/htbin-post/Entrez/nucleotide.html). This new reverse primer together with the *xylE* forward primer from Whyte *et al*, (1996), should have and did produce a 448 bp PCR product from amplified *P. putida* mt-2 DNA (Farrell and Kieser) and from 7/167 DNA (Figure 3.10). DNA isolated from 8/46 was not amplified using these *xylE* primers and no PCR product was generated. The *xylE* PCR product from 7/167 was not sequenced because not enough DNA was generated for a sequencing reaction to be performed. Based on PCR product size it appears that 7/167 and *P. putida* mt-2 to contain a similar region of the *xylE* gene whereas isolate 8/46 does not. This comparison links back to the plasmid DNA restriction digests, which also indicate that plasmids in 7/167 and 8/46 differ, and 7/167 and pWWO plasmids are similar.

Both Antarctic isolates, 7/167 and 8/46, contain a gene region with significant sequence homology to the *xylB* (benzyl alcohol dehydrogenase) of the archetypal TOL toluene degradative plasmid, pWWO, from *P. putida* mt-2. PCR and sequence results suggest that the x*ylB* degradative gene, and likely other genes involved in toluene degradation in mesophilic bacteria ie, *P. putida* mt-2, also occur in psychrotrophic Antarctic toluene-degrading *Pseudomonas* species.

4.4.2 Toluene Degradative Enzymes

To confirm the type of toluene degradative pathway (*meta* or *ortho*) present in Antarctic isolates 7/167 and 8/46, and to assess the presence of toluene degrading enzymes known to be involved in TOL plasmid mediated toluene degradation, enzyme assays were performed on cell-free extracts of 7/167 and 8/46, grown on 5 mM *meta*-toluate as a sole carbon source at 15°C.

Cell-free extracts were prepared from liquid cultures of 7/167 and 8/46 grown to early and late log phase growth. This was done primarily to ensure that any enzyme activity would be detected but also to compare enzyme activities over exponential growth range. As stated cells were grown on *meta*-toluate as a carbon source, due to their lack of growth on toluene in liquid medium. Although *meta*-toluate is not a substrate of the TOL plasmid encoded upper toluene degradation pathway it has been found to induce the upper pathway at low levels (Worsley and Williams, 1975). *Meta*-toluate is a lower (*meta*) toluene degradative pathway substrate and inducer (Worsley and Williams, 1975). The degradative enzymes assayed were benzyl alcohol dehydrogenase, benzaldehyde dehydrogenase, catechol 2,3-dioxygenase, and catechol 1,2-dioxygenase. Both benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase, are coded for by the upper pathway of TOL plasmids (Burlage *et al*, 1989). Catechol 2,3-dioxygenase is coded by the lower (*meta*) pathway of TOL plasmids and cleaves catechol in the *meta* position (Burlage *et al*, 1989). Catechol 1,2-dioxygenase is chromosomally encoded and cleaves catechol in the *ortho* position (Burlage *et al*, 1989).

Assay results of both early and late log phase cell-free extracts from 7/167 and 8/46 revealed that both benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase are induced by cell growth on *meta*-toluate and are present and catalytically active in both isolates. The per cell activity of benzaldehyde dehydrogenase decreased from early to late log phase cell growth in both isolates. In contrast the activity of benzyl alcohol dehydrogenase remained similar in early and late log phase 7/167 cells, but decreased in isolate 8/46. Comparing assays of early and late log phase cultures indicated that benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase were either more active or expressed in a greater abundance at the early log phase of growth in both 7/167 and 8/46. The decrease in enzyme activity over growth time was maybe due to a decrease in the cellular quantity of the enzymes. A decrease in enzyme levels could be due to the metabolism of *meta*-toluate (inducer) which may cause a reduction in its' enzyme induction capacity. However, enzymes were not purified beyond crude cell extract stage and thus no conclusions can be drawn as to the quantity of these degradative enzymes assayed. Toluene degrading Antarctic isolates, 7/167 and 8/46,

express toluene degrading upper pathway enzymes when grown on *meta*-toluate at 15°C, albeit at low levels.

The literature states that *P. putida* mt-2, when grown on 5 mM *meta*-toluate, has the following relative specific activities; benzyl alcohol dehydrogenase 0.031 μmol/min/mg protein, benzaldehyde dehydrogenase 0.023 μmol/min/mg protein, catechol 1,2-dioxygenase 0.009 μmol/min/mg protein catechol 2,3-dioxygenase 3.3 μmol/min/mg protein (Worsley and Williams, 1975).

Catechol 2,3-dioxygenase and catechol 1,2-dioxygenase were assayed to assess the mechanism of ring cleavage (*meta* or *ortho*) used by Antarctic isolates 7/167 and 8/46 when grown on *meta*-toluate as a sole carbon source at 15°C. Catechol 2,3-dioxygenase cleaves catechol in the *meta* position to produce a yellow coloured product, 2-hydroxymuconic semialdehyde (Bugg and Winfield, 1998). This enzyme is plasmid encoded in *Pseudomonas* species (Chatfield and Williams, 1986; Burlage *et al,* 1989). Catechol 1,2-dioxygenase cleaves catechol in the *ortho* position to produce a colourless product *cis, cis*,-muconate (Chatfield and Williams, 1986; Burlage *et al,* 1989; van der Meer *et al*, 1992). This enzyme is chromosomally encoded in *Pseudomonas* species (Burlage *et al*, 1989). Assay results indicate that when grown on *meta*-toluate Antarctic isolates cleave catechol in the *meta* position to produce 2-hydroxymuconic semialdehyde. Catechol 1,2-dioxygense was not active in 7/167 and 8/46. Results indicate that Antarctic isolates, 7/167 and 8/46, grown on *meta*-toluate express the *meta* cleavage enzyme, catechol 2,3-dioxygenase, cleave catechol in the *meta* position and thus metabolise *meta*-toluate via the plasmid encoded toluene degradative pathway. This is pathway is encoded by TOL type plasmids and is the commonest route for toluene degradation in pseudomonads. It can be proposed that these two Antarctic isolates contain TOL type toluene degradative plasmids. Curing the Antarctic isolates to remove any plasmids and assessing the cured isolates ability to grow on *meta*-toluate or toluene would confirm the location of the degradative genes, whether they be plasmid or chromosomally encoded. Probing Southern blots of Antarctic isolate DNA with labelled TOL plasmid fragments or with *xyl* genes, known to be involved in plasmid mediated toluene degradation, would confirm the existence

of TOL plasmids in Antarctic isolates and also give an indication of the presence of any TOL toluene degradative pathway genes in these isolates.

Comparison of catechol 2,3-dioxygenase activity in 7/167 and 8/46 shows 7/167 to have higher *meta* cleavage activity per cell than 8/46. A possible explanation for higher catechol 2,3-dioxygenase activity in 7/167 could be the presence of multiple genes encoding catechol 2,3-dioxgenases and therefore the expression of a higher quantity of *meta*-cleavage enzyme. Regions of the TOL plasmid are known to undergo spontaneous recombination with the chromosome and a 56 kb region, including the degradative pathway genes, found on TOL type plasmids has been identified as a transposable element (Frantz and Chakrabarty, 1986). It is possible that 7/167 contains duplicate copies of the gene encoding catechol 2,3-dioxygenase. Chatfield and Williams (1986) isolated a TOL plasmid from *Pseudomonas* strains that carry genes for two non-homologous *meta*-cleavage dioxygenases. They also isolated TOL plasmids that possessed two closely related catechol 2,3-dioxygenase genes that exhibit strong homology to each other and to the *xylE* gene of pWWO. The possible presence of multiple plasmids in 7/167 could also indicate that more than one catechol 2,3-dioxygenase gene could present. Total purification and quantification of catechol 2,3-dioxygenase from 7/167 and 8/46 would clarify whether this enzyme is more abundant or just more active in 7/167 compared to 8/46. Probing 7/167 and 8/46 total DNA with a labelled *xylE* gene probe could detect the presence of multiple *xylE* genes in isolate 7/167.

The presence and activity of the two upper toluene degradation pathway enzymes, benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase, and the lower (*meta*) pathway enzyme, catechol 2,3-dioxygenase, along with the presence of large plasmids in these isolates, indicates that 7/167 and 8/46 contain and express a plasmid encoded TOL toluene degradative plasmid. These two psychrotrophic Antarctic *Pseudomonas* species likely degrade toluene through a pathway analogous to the toluene degradative pathway encoded by the archetypal TOL plasmid, pWWO, in the mesophilic bacterium *P. putida* mt-2.

Chapter 5
Conclusions

5.1 Conclusions

Indigenous toluene and naphthalene degrading Antarctic bacteria, have been isolated from JP8 jet fuel contaminated soils at Scott Base, Antarctica. The isolates are able to grow on toluene and/or *meta*-toluate or naphthalene as a sole carbon source. The use of indigenous hydrocarbon degrading Antarctic bacteria to develop a bioremediation programme to clean up JP8 jet fuel contaminated soils in Antarctica has been proposed. Two toluene degrading isolates selected for in-depth study, 7/167 and 8/46, were isolated from different hydrocarbon contaminated sites at Scott Base, Antarctica. Both strains were able to grow in carbon-free liquid media supplied with *meta*-toluate as a sole carbon source, and were able to sustain growth on *meta*-toluate at temperatures ranging from 6 to 25°C. Based on growth ranges these isolates, 8/46 and 7/167, have been classified as psychrotrophs, and have optimum temperatures of 15°C and 20-25°C, respectively. The isolates are able to sustain growth on *meta*-toluate at temperatures typical of soil temperatures occurring at Scott Base in the summer months. Based on growth characteristics, these isolates would be expected to grow in JP8 jet fuel contaminated Scott Base soils and degrade components JP8 jet fuel when the soil temperature reached $\geq$6°C, and possibly lower temperatures, over the Antarctic summer months. It can be concluded that these isolates have the potential to be used to develop a successful bioremediation programme to clean up JP8 jet fuel contaminated soils at Scott Base, Antarctica.

Large plasmids, 61 kb and 95 kb, were isolated from 7/167 and 8/46, respectively. The plasmids isolated from 7/167 and 8/46 differ in size and fragment size and pattern. Isolate 7/167 plasmid DNA, when electrophoresed in an agarose gel and visualised with ethidium bromide, shows multiple bands, which could represent multiple plasmids, or multiple physical structures of a single plasmid, or both. In both total size and DNA fragments sizes, produced by the restriction enzyme XhoI, the plasmid DNA from 7/167 appears to be similar to the archetypal pWWO plasmid. The plasmid DNA

from isolate 8/46 is dissimilar in both total size and DNA fragments sizes, produced by the restriction enzyme XhoI, to both the 7/167 plasmid DNA and the pWWO plasmid. Analysis of the degradative genes in these Antarctic isolates revealed that at least one of their degradative gene sequences, (*xylB*), is homologous to a degradative gene found in the toluene degradative pathway encoded by the archetypal TOL plasmid, pWWO, of *P. putida (arvilla)* mt-2. Sequence analysis of a region of the *xylB* (benzyl alcohol dehydrogenase) gene indicates that 7/167, 8/46, and pWWO have a homologous DNA sequence in this region, 100%, 92%, and 99% homology, respectively.

Assays of four enzymes, involved in toluene degradation, catechol 1,2-dioxygenase, catechol 2,3-dioxygenase, benzyl alcohol dehydrogenase, and benzaldehyde dehydrogenase, from isolates 7/167 and 8/46 showed catechol 2,3-dioxygenase, benzyl alcohol dehydrogenase, and benzaldehyde dehydrogenase to be present and active. No activity was detected for catechol 1,2-dioxygenase. The substrate, catechol, was cleaved in the *meta* position by catechol 2,3-dioxygenase, to produce 2-hydroxymuconic semialdehyde. This mechanism of *meta* cleavage of catechol is encoded by the TOL toluene degradative pathway on TOL plasmids (Bugg and Winfield, 1998). The toluene and *meta*-toluate degradative mechanism used by Antarctic isolates', 7/167 and 8/46, appears analogous to the toluene degradative pathway encoded by the archetypal TOL plasmid, pWWO, in the mesophilic bacterium *P. putida* mt-2. It can be proposed that these Antarctic isolates encode a TOL toluene degradative pathway on a TOL type plasmid.

The differences in temperature growth ranges and plasmid size indicates that isolate 7/167, isolated from a fuel leak near the Scott Base kitchen, and 8/46, isolated from a site of a leaking pipeline to Williams Field, may have some metabolic differences. However, it can be proposed that psychrotropic toluene degrading Antarctic isolates', 7/167 and 8/46, harbour their degradative genes on a TOL plasmid, as they cleave catechol in the *meta* position with catechol 2,3-dioxygenase, which typically encoded by TOL toluene degradative plasmids as opposed to chromosomally encoded. Both isolates are able to degrade *meta*-toluate as a sole carbon source and are able to grow at low temperatures, similar to those found in soils at Scott base, Antarctica. It can be concluded that these two toluene degrading Antarctic *Pseudomonas* species have the

potential to be used in a bioremediation programme to clean up JP8 jet fuel contaminated soils at Scott Base, Antarctica.

5.2 Future Research

The following recommendations, by the candidate, elaborate on the research presented in this thesis and would provide further scientific knowledge concerning the potential of Antarctic hydrocarbon degrading *Pseudomonas* species to be utilised in a bioremediation programme in Antarctica.

To determine that toluene degrading isolates can metabolise toluene as a sole carbon source when grown in liquid media, the growth experiments should be repeated using a lower concentration of toluene. Measuring the concomitant decrease in toluene concentration, by high pressure liquid chromotography, and increase in cell biomass, spectrophotometrically, or measuring the evolution of radioactive CO_2 from metabolism of radioactive labelled toluene would confirm the degradative ability of Antarctic isolates.

Once cells are growing on toluene as a sole carbon source, assays of enzymes involved in toluene degradation should be repeated on cells grown at a low temperature (6°C). Purification of individual enzymes to be assayed and assaying these enzymes at a low temperature would give an indication of the activity of degradative enzymes at low temperatures and the rate of toluene degradation that could occur at low temperatures. This information would clarify the rate of toluene degradation that could occur in contaminated Antarctic soils.

Confirmation of the presence and type of toluene degradative pathway in Antarctic isolates, curing experiments should be done on these isolates. Curing the cells of their plasmid(s) would determine equivocally if the toluene degradative pathway is located on the plasmid or the chromosome. Probing Southern blots of digested Antarctic DNA with radioactively labelled TOL plasmid DNA would clarify whether TOL type degradative plasmids are present in Antarctic toluene degrading isolates. Amplification of Antarctic degradative gene sequences with primers designed from

known toluene degradative genes and sequence analysis of PCR products would confirm the homology of toluene degradative genes in Antarctic isolates to archetypal toluene degradative gens in Northern Hemisphere bacteria.

To further assess the potential of toluene degrading Antarctic bacteria to be used to develop a bioremediation programme to clean up JP8 jet fuel contaminated in Antarctic soils growth parameter need to be determined. The pH of the soils these bacteria were isolated from is high (pH~9). Determination of the effect of pH on isolates' ability to degrade components of JP8 jet fuel is needed to assess the ability of these isolates to degrade hydrocarbons under these conditions. Assessment of the maximum and minimum concentration of hydrocarbons required to sustain isolate growth, as well as assessment of various limiting abiotic factors in the contaminated site would be required to ensure the site is conducive to microbial degradation of the contaminant.

Appendices

Appendix A

Description of solid media used in this study.

Solid Media	Purpose	Components (per litre)	Recipe
R2A Agar (Difco) **(R2A)**	Nutrient rich solid media for selection of and enumeration of heterotrophic organisms	Bacto Yeast Extact 0.5g Bacto Proteose Peptone No.3 0.5g Bacto Casamino Acids 0.5g Bacto Dextrose 0.5g Soluble Starch 0.5g Sodium Pyruvate 0.3g Potassium Phosphate Dibasic 0.3g Magnesium Sulphate 0.05g Bacto Agar 15g	Dissolve 18.2g in 1L distilled water
Bushnell Haas Broth (Difco) solidified with purified agar (Oxoid) **(BHA)**	Selective solid minimal salt media for selection of hydrocarbon utilising bacteria	Magnesium Sulphate 0.2g Calcium Chloride 0.02g Monopotassium Phosphate 1g Ammonium Phosphate Dibasic 1g Potassium Nitrate 1g Ferric Chloride 0.05g	Mix 500ml of sterile double strength broth (3.27g in 500ml) to 500ml of sterile double strength purified agar (16g in 500ml).
Bushnell Haas agar plates amended with *meta*-toluate (Aldrich) **(BHA-mtol)**	Selection of *meta*-toluate-degrading isolates	Bushnell Hass solidified as described above *meta*-toluate 136.15g	Dissolve 136.15g of *meta*-toluate in 1.2M Sodium Hydroxide and sterilise with a 0.2µm filter (Gelman Sciences). Add 5ml to 1L BHA to give a final concentration of 5mM *meta*-toluate.

Description of liquid media used in this study.

Liquid Media	Purpose	Components (per litre)	Recipe
½ Plate Count Broth (Difco) (**½PC**)	Nutrient rich media for enumeration of heterotrophic organisms	Bacto Yeast Extract 5g Bacto Tryptone 10g Bacto Dextrose 2g	Dissolve 8.5g of Plate Count broth in 1L of distilled water.
Bushnell Haas Broth (Difco) (**BH**)	Minimal salt media or studying microbial utilisation of hydrocarbon	Magnesium Sulphate 0.2g Calcium Chloride 0.02g Monopotassium Phosphate 1g Ammonium Phosphate Dibasic 1g Potassium Nitrate 1g Ferric Chloride 0.05g	Dissolve 3.27g in 1L of distilled water.

Appendix B

4/2 Phosphate buffer	**g/L**
Na_2HPO_4	4g
KH_2PO_4	2g

Dissolve chemicals in 1L of distilled water and adjust to pH7.5 with 10M NaOH. Autoclave to sterilise

Tris-EDTA buffer	**g/L**
Tris chloride	1.57g
EDTA	0.29g

Dissolve chemicals in 1L of distilled water and adjust to pH8 with 10M NaOH. Autoclave to sterilise

Tris-acetate/EDTA electrophoresis buffer (TAE Buffer) (50X stock)	**g/L**
Tris base	242g
Glacial acetic acid	57.1ml
0.5M EDTA	100ml

Dissolve chemicals in 1L of distilled water and adjust to pH8 with 10M NaOH. Autoclave to sterilise

DNA Loading Dye	**g/L**
Xylene cyanol	0.05g
Glycerol	5ml
EDTA	0.186g
Bromo Phenol Blue	0.05g
1xTAE	10ml

Appendix C

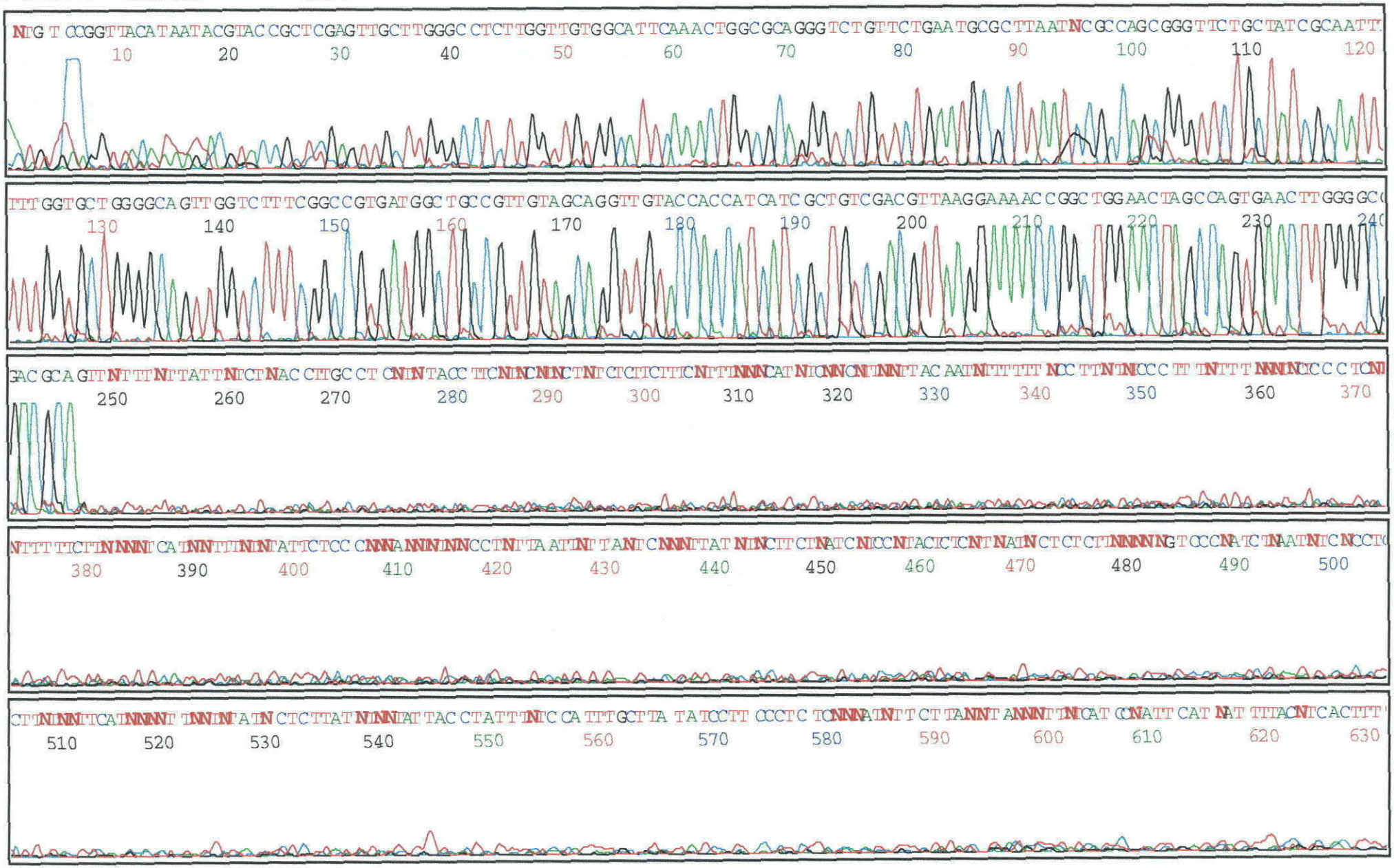
Chromosomal DNA Shearing Method for Plasmid Isolation

The large scale preparation of plasmid DNA by the alkaline lysis method (Sambrook *et al*, 1989a) were used and the plasmid DNA was separated from the chromosomal DNA via a cesium chloride gradient. The plasmid DNA band could not be isolated from the linear chromosomal DNA. In order to remove the linear chromosomal DNA from the plasmid DNA, the large scale plasmid isolation method of Sambrook *et al* (1989a) was modified. The cesium chloride gradient step was omitted and the DNA preparation was sheared with a 21 gauge needle by gently drawing and emitting into a syringe. Samples of the preparation were digested for 30 and 60 minutes with DNAse. Theoretically long pieces of linear chromosomal would be broken down into small lengths and digested by the DNAse, whereas the covalently closed circular (CCC) plasmid DNA would be protected from the shearing force of the method and the DNAse treatment.

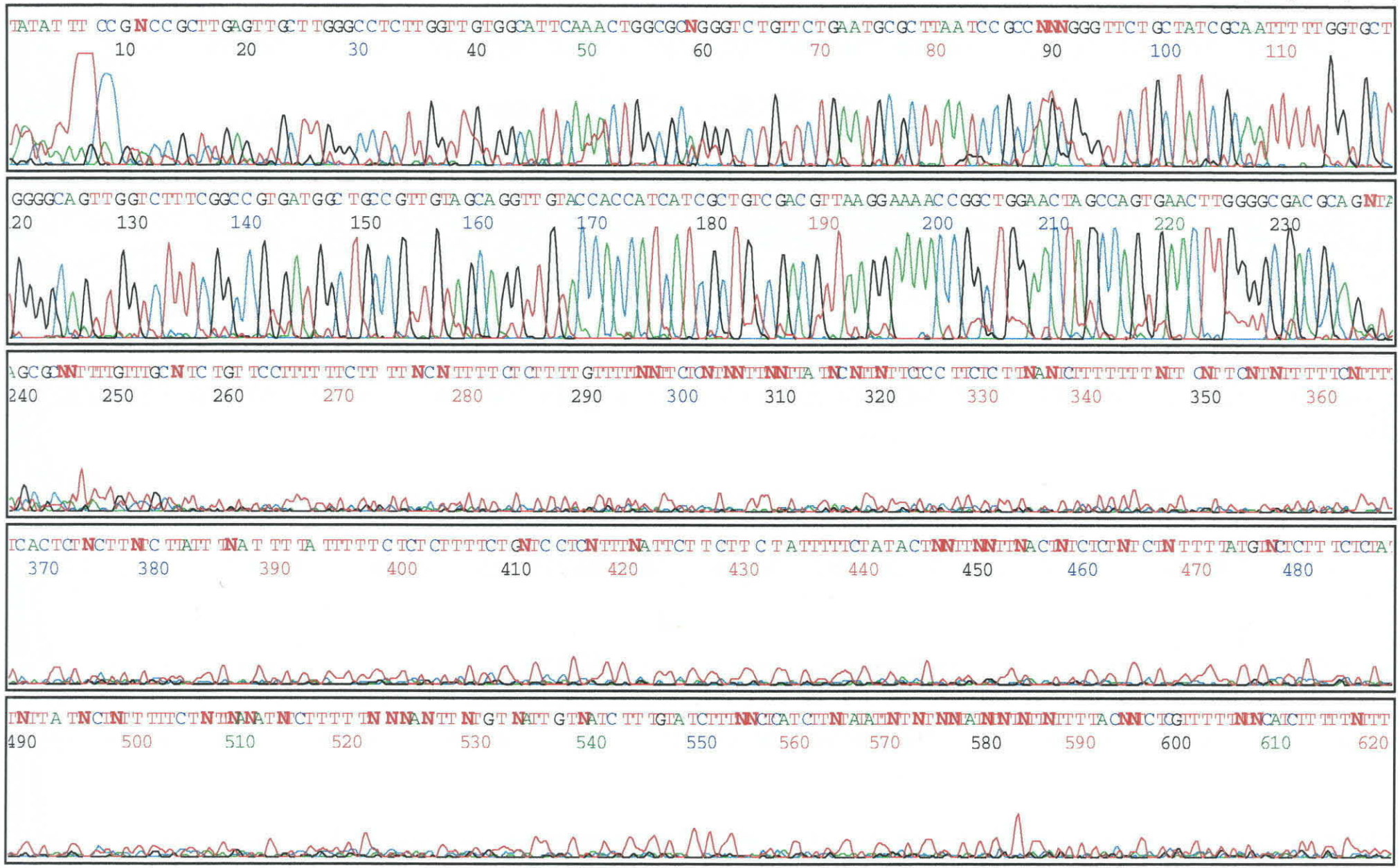
Electrophoresis Well Method for Plasmid Isolation

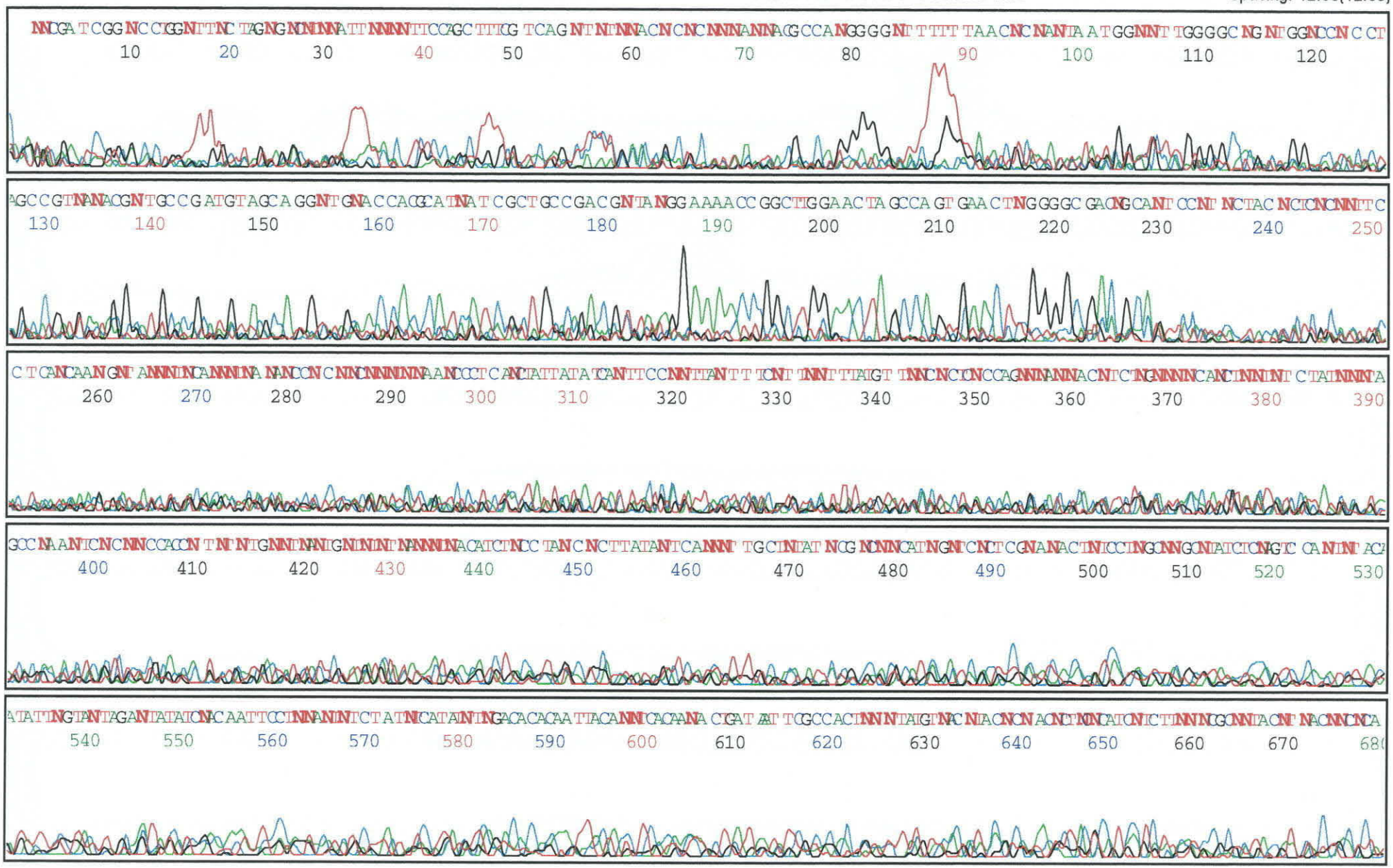
To isolate plasmid DNA, contaminated with linear chromosomal DNA, isolated by the Farrell method an agarose gel electrophoresis method was used. Total DNA was electrophoresed on a 1% agarose gel in TAE buffer. Approximately 2ml of the DNA preparation was loaded into wells across the length of the gel and separated by electrophoresis. The plasmid DNA bands (open circular plasmid DNA, CCC plasmid DNA, and linear plasmid DNA) migrated unique distances on the agarose gel and were thus separated from each other and from the linear chromosomal DNA smear. Each band of DNA was then excised, using a sterile razor blade, from the agarose gel. The excised agarose strip, containing a band of DNA, was set into a fresh agarose. A well was cut into the fresh gel directly below the DNA containing inserted gel strip and the well was filled with electrophoresis buffer (TAE). A voltage was passed through the gel and the DNA in the gel strip was run into the buffer in the well. The buffer containing DNA was collected using a sterile pipette. The DNA was ethanol precipitated at-20°C overnight, harvested and resuspended in Tris EDTA buffer (pH8).

Appendix D DNA Sequence for *xylB* gene region from *P. putida* mt-2



DNA Sequence for *xylB* gene region from Antarctic Isolate 7/167




ABI PRISM
Model 377
Version 3.3
SemiAdaptive
Version 3.2
07•8/46/F2
813
8/46/F2
Lane 7
Signal G:12 A:13 T:16 C:19
DT {BD Set Any-Primer}
dRhod
Points 1453 to 11804 Pk 1 Loc: 1453
Page 1 of 3
Wed, 4 Nov 1998 10:06 AM
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