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Characterization of Pyrene Degradation by *Mycobacterium* sp. Strain S65

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ABSTRACT

The microbial degradation of pyrene, a 4-ring polycyclic aromatic hydrocarbon (PAH), has been elucidated with the increasing number of pyrene-degrading bacteria that have been isolated in recent years. A pyrene degrading bacterium identified as *Mycobacterium* sp. strain S65, was isolated from a jet-fuel contaminated site in Sept-Iles, northern Quebec, Canada. S65 utilized pyrene, phenanthrene, and fluoranthene as sole carbon and energy sources, but did not degrade naphthalene, anthracene, and fluorene. Pyrene mineralization was enhanced by adding benz[a]anthracene, benzo[a]pyrene, or phenanthrene as cosubstrates. When added to PAH contaminated soil as a potential bioaugmentation agent, S65 did not appear to survive well, nor was it effective at degrading PAHs under these conditions.

Pyrene catabolic genes in S65 were partially characterized by Southern hybridization using a probe constructed from the naphthalene inducible pyrene dioxygenase gene, *nidA*, from the pyrene-degrading bacterium, *Mycobacterium* sp. strain PYR-1. The hybridization results indicated that *nidA* homologues were found in more than one location in S65. Two positive clones were obtained; five open reading frames (orfs) and seven orfs were predicted from clone 1 and clone 2, respectively. Two putative bacterial ring-hydroxylating dioxygenase genes were found in each clone. Only *orf1* of clone 1 showed high homology (99.5%) to the *nidA* gene. Sequence similarity between the *nidA* gene and the *nidA* homologue in clone 2, designated *pdoA*, decreased to 89%. By BlastN search, the second dioxygenase gene in both clones had no nucleotide sequence similarity to any known dioxygenase genes in the databases. The deduced amino acid sequence of the second dioxygenase gene showed high homology to a hypothetical dioxygenase from a dioxin degrading bacterium, *Sphingomonas* sp. strain RW1. Two alcohol dehydrogenase genes were predicted downstream of the *nidA* and *pdoA* genes in both clones. The one closest to the *nidA/pdoA* gene was predicted to function as a *cis*-diol dehydrogenase because it belongs to the short-chain alcohol dehydrogenase family as do other *cis*-diol dehydrogenases. The other alcohol dehydrogenase gene belongs to the zinc-containing alcohol dehydrogenase family and is suspected to be involved in the dehydrogenation of benzyl alcohol to benzaldehyde. This

hypothesis was supported by the fact that benzyl aldehyde is one of the metabolites in pyrene catabolism in another pyrene degrading bacterium, *Mycobacterium* sp. strain KR2.

RESUMÉ

La dégradation microbienne du pyrène, un hydrocarbure aromatique polycyclique (HAP), est maintenant bien caractérisée suite à l'isolement, au cours des dernières années, d'un grand nombre de bactéries capables de dégrader le pyrène. Une bactérie capable de dégrader le pyrène de part son habileté à dégrader le phénanthrène et identifiée comme étant un *Mycobacterium sp.,* souche S65, a été isolée sur un site contaminé par du carburant d'avion situé à Sept-Îles dans le nord du Québec au Canada. S65 est capable d'utiliser le pyrène, le phénanthrène et le fluoranthène comme sources de carbone et d'énergie mais n'est pas en mesure de dégrader le naphtalène, l'anthracène et le fluorène. La minéralisation du pyrène est favorisée par l'ajout d'un co-substrat tel le benz(a)anthracène, le benzo(a)pyrène ou le phénanthrène. Lorsque S65 est ajoutée à un sol contaminé en HAP comme agent potentiel de bioaugmentation, celle-ci ne semble pas avoir un taux élevé de survie ni dégrader efficacement les HAPs sous ces conditions.

Les gènes responsables du catabolisme du pyrène chez S65 ont été partiellement caractérisés par hybridation d'un transfert d'ADN par buvardage (Southern) avec la sonde *nidA.* Cette sonde fut construite à partir du gène de la dioxygénase du pyrène inductible par le naphtalène chez *Mycobacterium sp.,* souche PYR-1 dont la protéine est capable de dégrader le pyrène. Les résultats de l'hybridation ont démontré la présence d'homologues de *nidA* en plusieurs emplacements chez S65. Deux clones positifs obtenus, les numéros 1 et 2, possèdent respectivement cinq et sept cadres de lecture ouverts (ORFs). Deux gènes codant potentiellement pour des dioxygénases ayant une activité d'hydroxylation de noyaux sont présents au niveau de chacun des clones. Seul l' *ORF1* présent sur le clone 1 présente un pourcentage d'homologie élevé avec *nidA* (99.5%). L'homologie de séquence entre *nidA* et son homologue présent sur le clone2, designé *pdoA* n'est que de 89%. L'analyse de sequences par BlastN a révélé que la seconde dioxygénase se retrouvant au niveau des clones 1 et 2 ne possède aucune homologie de séquence nucléotidique avec les gènes de dioxygénases disponibles dans les bases de données. Par contre, la séquence en acides aminés déduite pour la seconde dioxygénase présente une homologie élevée avec une dioxygénase hypothétique du *Sphingomonas* sp., souche

RW1, capable de dégrader les dioxines. Pour les deux clones, deux gènes codant pour des déshydrogénases d'alcool ont été identifiés en aval des gènes *nidA* et *pdoA*. Le gène situé le plus près de *nidA/pdoA* est présumé fonctionner comme une déshydrogénase cis-diol car il appartient à une famille de déshydrogénase d'alcool à courte chaîne tout comme d'autres déshydrogénases cis-diol. L'autre gène de déshydrogénase d'alcool appartient à la famille de déshydrogénases d'alcool renfermant du zinc comme élément structural et est présumé avoir une activité de déshydrogénation permettant la conversion de l'alcool benzylique en benzaldéhyde. Cette hypothèse est supportée par le fait que le benzaldéhyde est l'un des métabolites dans le catabolisme du pyrène chez le *Mycobacterium sp.*, souche KR2, capable de dégrader le pyrène.

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous organic pollutants in nature. PAHs are mainly produced as a result of incomplete combustion of organic materials such as fossil fuels, by-products of mobile exhaust, cigarette smoke, and during cooking. PAHs are considered as health hazards because some of them possess mutagenic, genotoxic, and/or carcinogenic activities. The degree of toxicity of PAHs increases proportionally to the number of aromatic rings (Binkova *et al.* 2000; Marston *et al.* 2001). For example, 3-ring PAHs such as anthracene and phenanthrene do not have any activities in bacterial mutagenicity, carcinogenicity, and DNA adduct formation. On the other hand, the 4-ring PAH, pyrene, showed mutagenic activity in bacteria, and the 5-ring PAH, benzo[a]pyrene, showed very high carcinogenic activity in mouse skin (Ingram *et al.* 2000).

A pyrene degrading bacterium, identified as *Mycobacterium* sp. strain S65, was isolated from a jet-fuel contaminated site in Sept-Iles, northern Quebec, Canada. S65 utilized pyrene (60% mineralization), phenanthrene (60% mineralization), and fluoranthene (20% mineralization) as sole carbon and energy sources, but did not degrade naphthalene, anthracene, and fluorene. Pyrene mineralization was enhanced by adding benz[a]anthracene, benzo[a]pyrene, or phenanthrene as cosubstrates. Based on the mineralization results, extensive molecular analyses on pyrene catabolic genes in S65 were carried out by Southern hybridisation using a pyrene dioxygenase, *nidA* gene probe, characterized in another pyrene-degrading bacterium, *Mycobacterium* sp. strain PYR-1, subsequently by cloning and sequencing of the clones possessing the pyrene catabolic genes.

The pyrene degrading bacterium, *Mycobacterium* sp. strain S65 was introduced, as a bioaugmentation agent, into a soil historically contaminated with polycyclic aromatic hydrocarbons (PAHs). The bioaugmentation study was continued for 90 days, and six different treatments were tested to determine optimum degradation kinetics for S65 in the soil. The survival of S65 in the soil was determined using a combination denaturing gradient gel electrophoresis (DGGE)-hybridization technique.

Chapter 1. Literature Review

Over the last 150 years, the concentration of polycyclic aromatic hydrocarbons (PAHs) in soils has drastically increased in industralized countries caused largely by human activities (Wilson and Jones 1993). Public concern over the hazardous effects of PAHs have increased in the past several decades because some PAHs exert mutagenic, genotoxic, and carcinogenic effects on various organisms, including humans. PAHs are ubiquitous environmental pollutants and substantial amounts of these compounds in the environment may result in potential adverse health effects to humans and alteration of ecosystems (Binkova *et al.* 2000; Bozall and Maltby 1997; Marston *et al.* 2001).

1.1. Polycyclic aromatic hydrocarbons (PAHs)

1.1.1. Physical and chemical properties of PAHs

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds which consist of two or more fused benzene rings in linear, angular, or cluster arrangements with the linear form being the most unstable and the angular form the most stable conformation. PAHs only contain carbon and hydrogen atoms and are called aromatic because some of the carbon atoms are doubly bonded to other carbon atoms. (Blumer 1976). PAHs with 3 fused-benzene rings or less are considered low-molecular-weight (LMW) PAHs, and those with 4 or more fused-benzene rings are high-molecular-weight (HMW) PAHs.

In general, PAHs are non-polar, electrophilic, nucleophilic compounds, and are highly hydrophobic in nature. The solubility of PAHs inversely correlates to the increasing number of fused rings. For example, the aqueous solubility of naphthalene (2-fused rings) is 31.7 mg/L compared to that of phenanthrene (3-fused rings), 1.29 mg/L, and pyrene (4-fused rings), 0.14 mg/L (Molina *et al.* 1999). Volatility also decreases with increasing molecular size and angularity of a PAH molecule due to an increase in stability of the aromatic rings. These physico-chemical properties are the primary factors contributing to the recalcitrance and persistence of PAHs, especially HMW-PAHs in the environment.

1.1.2. Sources

A number of sources of PAHs have been identified. The sources vary from possible biosynthesis by plants and microorganisms, natural activities (i.e., forest fires and volcanos), to anthropogenic activities (i.e., power generation, coke production, petroleum catalytic cracking, incineration, carbon black production, etc.). A rapid increase in the concentration of PAHs in the environment, however, is caused by anthropogenic sources and is suspected to increase the incidences of carcinogenesis and mutagenesis in organisms living in the environment, exceeding the tolerance level.

Certain bacteria, fungi, plants, and animals have been shown to synthesize a variety of polycyclic quinone pigments, which may be transformed into PAHs during carbonization processes over long periods of time. The evidence for PAH biosynthesis is as yet, inconclusive. It is, however, accepted that some biosynthesis of PAHs may occur, but the concentration of PAHs released to the environment by biosynthesis is insufficient to give an adverse impact on ecosystems (Environment Ontario 1992).

Another natural source of PAHs is forest fires and it is one of the major sources of PAH production. There are annually 4,367 incidences of forest fires in average in the past 10 years, and over 1.2 million hectares of forests were burned in Canada (Canadian Forest Service http://www.nrcan-rncan.gc.ca/cfs-scf/index_e.html).

The combustion or pyrolysis of organic materials, by far, releases the greatest amounts of PAHs into the environment in a short period of time. The anthropogenic processes include the combustion of fossil fuel, industrial and domestic heating, production of pyrolysis products (coal tar, creosotes, and carbon black), and vehicle exhausts. Domestic activities such as cigarette smoke and grilled foods are also common sources of PAHs and may have a greater impact on humans. The major sources of PAHs released into the environment are summarized in Table 1.1.

Table 1.1. Major sources and transport mechanisms of PAHs in the environment.

Natural oil seeps
Refinery and oil storage wastes
Accidental spills from oil tankers and other ships
Municipal and urban wastewater discharge runoff
River-borne pollution
Atmospheric fallout of fly ash particulates
Petrochemical industrial effluents
Coal tar and other coal processing wastes
Automobile engine exhausts
Combustion of fossil fuels (gasoline, kerosene, coal, diesel fuel)
Smoked, charcoal broiled, or pan fried food
Tobacco and cigarette smoke
Forest and prairie fires
Rural and urban sewage sludge
Refuse and waste incineration
Coal gasification and liquifaction processes
Creosote and other wood preservative wastes
Commercial and pleasure boating activities

Reference: IARC (1972-1990) Monographs on the Evaluation of Carcinogenic Risks to Humans, Vol 1-49, International Agency for Research in Cancer, Lyons.

Crude petroleum and many petroleum products contain a complex mixture of organic compounds including PAHs. PAHs are often released from crude oils during catalytic cracking (breaking up of long chain hydrocarbons) to derive fuel oil and other petroleum products, such as asphalt. In 1986, the estimated annual PAH production from petroleum refineries in Ontario ranged from 66.7 to 648 kg. (Environment Ontario 1992).

Pyrolysis products such as crude coal tars, carbon black, and heavy coal distillates are another major source and produce many kinds of PAHs. Nishioka *et al.* (1986) determined 48 different kinds of PAHs in the heavy coal distillates, coal tar, and carbon black samples from a coal-mining site in the U.S.A. Ten PAHs, detected in coal tar, and 6 PAHs, in carbon black, were considered as priority pollutants due to their abundance and toxicity by the US Environmental Protection Agency (EPA). The sixteen PAH priority pollutants are listed in Table 1.2.

Table 1.2. US EPA 16 priority PAHs

	Threshold of PAH contaminants in soil in Ontario, Canada (μg/g of soil)
Naphthalene	40
Acenaphthylene	840
Acenaphthene	1300
Fluorene	350
Phenanthrene	40
Anthracene	28
Fluoranthene	40
Pyrene	250
Chrysene	19
Benz[a]anthracene	NA
Benzo[a]pyrene	1.9
Benzo[b]fluoranthene	19
Benzo[k]fluoranthene	19
Benzo[ghi]perylene	40
Dibenz[ah]anthracene	1.9
Indeno[1,2,3cd]pyrene	19

Creosotes are also a significant source of PAHs released into the environment. They are derived from coal tar and have been extensively used as wood preservatives. They contain up to 85 – 90 % PAHs (Cerniglia 1992) and contaminate soils and groundwater through routine operational spillage and losses from storage areas (Environment Ontario 1992).

Considerable amounts of PAHs are released from motor vehicle exhaust into the atmosphere (Lim *et al.* 1999; Marr *et al.* 1999). In the United States, PAHs released to the atmosphere from motor vehicles account for one-third of total PAH emissions. Marr *et al.* (1999) tested the amount of PAHs produced during the combustion of various gasoline brands. Their study demonstrated that individual brands have different PAH compositions and emission levels. On average, naphthalene contributed 97% of PAHs measured in all the gasoline brands, with one brand consisting of 2600 mg of naphthalene per L of gasoline. Their study also found that HMW-PAHs are dominant PAHs in particulate-phase emissions and the particulate PAHs are considered as major contributors to some allergic reactions, such as asthma.

1.1.3. Environmental impacts

PAHs are ubiquitous in both aqueous and terrestrial ecosystems and are found in high concentrations on many industrial sites and just downstream of industrial effluent discharges. The high concentration and toxicity of these PAHs raise a tremendous concern as to their adverse effects on population dynamics in ecosystems (Fernandes *et al.* 1999; Jiries *et al.* 2000; Maltby *et al.* 1995; Zeng and Vista 1997).

Zeng and Vista (1997) analyzed the effluent discharged from a wastewater treatment plant in San Diego and detected up to 17.5 mg/g of particulate PAHs in the effluent samples. Particulate PAH concentrations in sediments collected nearby in the San Diego Bay area also showed high PAH contents; at one sampling site, 120 mg/g of total organic carbon in the sediment was attributed to PAHs. In the same study, LMW-PAHs were the predominant PAH in the aqueous samples (38 – 100% of total PAHs) whereas HMW-PAHs predominated in the sediment samples. High concentrations of these PAHs in water and sediments have affectedthe diversity of organisms, especially the structure and functioning of benthic communities.

Interest in the biological impact of PAHs on environments and organisms increased significantly after the discovery of the carcinogenic properties of benzo[a]pyrene in the mid-1930's. It was the first indication that disease could be caused by simple organic molecules, and not by microorganisms. Since then, numerous studies have been conducted on the toxicity of individual contaminants, industrial effluents, and sediments on various organisms (Geffard *et al.* 2001; Landrum 1989; Pickering 2000).

The US Environmental Protection Agency (EPA) compiled 16 PAHs as priority pollutants (Table 1.2) based on their abundance in the environment and toxicity. The toxicity of various PAHs depends on their specific structures and positions of ring substituents (Nishioka *et al.* 1986). It is generally accepted that LMW-PAHs do not exert toxic activities as much as HMW-PAHs. Ingram *et al.* (2000) carried out mutagenicity assays in bacteria using various PAHs and showed that the LMW-PAHs did not induce mutagenic activity, while all the HMW-PAHs tested, (fluoranthene, pyrene, chrysene, benz[a]anthracene, and benzo[a]pyrene), induced high levels of mutagenicity. The same

results were obtained when a benthic microinvertebrate, *Gammarus pulex* was used as a test organism (Bozall and Maltby 1997). It was shown that three- to five-ring PAHs such as phenanthrene, fluoranthene, and pyrene were the major sources of toxicity to the organism.

LMW-PAHs may not have carcinogenic effects, however, they are still a concern as hazardous compounds. For example, exposure to naphthalene has been implicated in hemolytic anemia in newborn infants or in people with glucose-6-phosphate dehydrogenase deficiency (Shah and Santucci 2000). Also, the presence of weakly or non-carcinogenic PAHs in a complex mixture alters the activities of carcinogenic PAHs in rodents (Marston *et al.* 2001). These findings demonstrated that PAHs with less or no immediate toxic effects should not be ignored as potential toxic compounds.

Recently, many studies have implicated a correlation of PAH-induced carcinogenesis with the occurrence of lung, skin, and breast cancers in humans (Ingram *et al.* 2000; Rundle *et al.* 2000). In humans, PAHs are transformed into metabolites called epoxides by a monooxygenase, cytochrome P450. The epoxides covalently bind to molecules such as DNA, RNA, and proteins. This binding of epoxidated PAHs to DNA (PAH-DNA adduct) determines the level of carcinogenicity and interferes with DNA repair mechanisms, leading to increased tumorigenic responses.

Another toxicity study was conducted by Marston *et al.* (2001) to understand the correlation of the tumour initiating activity of a mixture of PAHs and individual PAHs in mouse skin. The study showed that 1 mg of a PAH cocktail was sufficient to cause a 13% mortality rate in mice in 25 weeks of promotion. In the case of individual PAHs such as benzo[a]pyrene, a similar mortality rate was observed at the lower concentration of 200 nmol (50.4 μg), in the same period of time. The presence of PAHs such as phenanthrene and pyrene alone did not show direct activities on the induction of tumour formation and cancer, but greatly enhanced DNA adduct formation by benzo[a]pyrene. This study clearly demonstrated that PAHs that are not individually toxic could be a trigger to increase the toxicity of highly toxic PAHs.

Because of the consequences of PAH residues as potential health hazards, their remediation from contaminated environments has received tremendous attention and has

become an important process to minimize the impact on organisms that may come into close contact with PAHs.

1.1.4. Remediation of PAH

PAHs can be remediated in many different ways. The possible fate of PAHs in the environment includes abiotic and biotic processes. Abiotic processes such as volatilization, photooxidation, chemical oxidation, adsorption, and leaching have no significant effect on the reduction of the concentration of HMW-PAHs and only account for a 2 – 20 % reduction in the concentration of LMW-PAHs in contaminated soils (Park *et al.* 1990). Biotic processes such as microbial degradation (bioremediation) and phytoremediation can degrade both LMW- and HMW-PAHs to a great extent, and are the processes primarily responsible for the removal of PAHs from soils. Biotic processes are the method of choice because they require the least applications of other chemicals, less disturbance of the sites, and are considered environmentally friendly.

Physico-chemical methods such as landfilling and soil washing are still used routinely to decontaminate industrial sites of PAHs (Head 1998). The problem with physico-chemical treatments such as landfilling is a limited available space for the storage of contaminated soils and the safety of the surrounding environment due to leakage of the contaminants from confined compartments. Another physico-chemical method, soil washing, extracts PAHs from the contaminated sites but does not destroy the PAHs. Soil incineration removes PAHs very efficiently from contaminated soils by heating the soils at extremely high temperatures and combustion of the organics. Soil incineration is fast and efficient at removing contaminants from the environment, but it could become very costly when the volume of contaminated material increases and it destroys the biological systems in the soils.

Bioremediation is a process that uses microorganisms to break down environmental pollutants to less toxic and water-soluble compounds with relatively little disturbance to biological systems. Because PAHs only consist of carbon and hydrogen, when the complete mineralization of PAHs occurs only carbon dioxide and water are formed as end products. The complete mineralization of PAHs is an attractive benefit

over the physico-chemical processes. Another advantage of bioremediation is that the decontaminated soils will maintain their biological systems intact (Head 1998).

In situ bioremediation is a method used to remove PAH contaminants in place. Contaminants are not excavated and transported to other sites and the contaminated site is relatively undisturbed during the treatment (Wilson and Jones 1993). The most common approach of *in situ* bioremediation is an addition of nutrients (mostly in the form of fertilizers), oxygen sources, and surfactants (Mishra *et al.* 2001). These amendments support and optimize conditions for the growth of potential PAH-degrading indigenous microorganisms.

In contrast, *ex situ* treatment requires the initial removal of contaminated soils prior to their treatment on-site or off-site (Marcoux *et al.* 2000). The best example is the use of biopiles or bioreactors. Contaminated samples are transferred to a reactor in which the optimum remediation conditions are maintained and monitored. The reactor can be supplied with PAH-degrading microbial consortia that were isolated from the samples and acclimatized with a mixture of PAHs of interest to increase the rates of degradation (Ericksson *et al.* 2000).

Major factors influencing the efficiency of bioremediation processes in soils are environmental, nutritional, and the bioavailability of PAHs. The main environmental factors are pH, salinity, temperature, moisture content, and soil properties (Table 1.3). For example, a more dense (clay) soil environment limits the mobibility of nutrients and oxygen to microorganisms, thus decreasing the efficiency of biodegradation. The moisture level of the soil also greatly influences microbial activity. Cattaneo *et al.* (1997) demonstrated that the degradation potential of *Burkholderia cepacia* BRI6001 on a pesticide, 2,4-D, was halted when the water holding capacity decreased below 15%.

Addition of inorganic nutrients such as nitrogen (N) and phosphorus (P), as a part of bioremediation processes, plays an important role in stimulating microbial growth, and metabolism. The lack of available N and P severely limits the extent of PAH degradation in nature. These nutrients need to be replenished constantly during treatment to maintain optimum conditions for the degradation of PAHs.

The bioavailability of PAHs is another important key for the success of bioremediation. For microorganisms to degrade PAHs, these substrates have to be readily accessible. In the soil environment, PAHs are strongly adsorbed onto soil particles and persist within the pores, which are too small for micoroorganisms to access. The high hydrophobicity of PAHs also causes unexpectedly slow biodegradation rates because microorganisms assimilate substrates primarily when they are dissolved in water. In order to overcome these limitations, surfactants are often applied to increase the solubility of PAHs (Tiehm 1994). There are three main mechanisms by which surfactants increase the bioavailability of PAHs: (i) an increase in contact area due to dispersion of nonaqueous-phase liquid which is caused by a reduction in the interfacial tension between the aqueous phase and the nonaqueous phase; (ii) formation of micelles into which PAHs are trapped, resulting in an increase in solubility of the PAHs; (iii) mass transfer of the PAHs from the solid phase to the aqueous phase, which is caused by decreasing the surface tension of the pore water in soil particles, interaction of the surfactant with solid interfaces, and contact of PAH with single surfactant molecules (Volkering *et al.* 1995).

Table 1.3. Environmental conditions affecting the degradation of organic contaminants in soil

Parameter	Conditions required for microbial activity	Optimum values for PAH degradation
Soil moisture	25-85% of water-holding capacity	30-90%
Soil pH	5.5-8.5	7.5-7.8
Oxygen	aerobic, minimum air-filled pore content space of 10%	10 – 40% O_2
Nutrient	N and P for microbial growth C:N:P 120:10:1	salt concentration <4% C:P 800:1, C:N 60:1
Temperature (°C)	15-45	24 – 30

(Wilson and Jones 1993)

1.2. PAH degrading bacteria

A wide variety of bacteria have the ability to breakdown PAHs. A greater number of PAH-degrading bacteria are isolated from soils, sediments, or aquatic systems contaminated with organic hydrocarbons (Ahn *et al.* 1999; Dagher *et al.* 1997; Iwabuchi

and Harayama 1997; Mueller *et al.* 1997) than from uncontaminated systems. This is because the adaptation of certain microorganisms for the available nutrient sources, PAHs, favours the population dynamics of the contaminated environment towards PAH-degrading bacteria.

1.2.1. The diversity of PAH-degrading bacteria

Numerous taxa of bacteria have been shown to have the potential to utilize PAHs. Many diverse organisms are capable of degrading LMW-PAHs (Table 1.4) such as naphthalene, anthracene, and phenanthrene. The majority of bacterial species isolated as LMW-PAH degrading bacteria are Gram-negative bacteria. The best known genus for LMW-PAH degradation is *Pseudomonas*. Various strains of one particular *Pseudomonas* species, *P. putida*, have been extensively studied for both the biochemical and genetic mechanisms of PAH degradation and are often referred to as model organisms (Cerniglia 1992; Kiyohara *et al.* 1994; Pflugmacher *et al.* 1996). Recently, an increasing number of cases of the isolation of non-pseumomonad PAH-degrading bacteria have been reported. The non-pseudomonad PAH degraders have been isolated on phenanthrene instead of naphthalene, which had been previously used to isolate PAH degrading bacteria. By increasing the substrate specificity, more selective pressure can be applied on the isolation process, minimizing the isolation of naphthalene degrading bacteria, which are more likely to be pseudomonads.

Little information exists on the microbial degradation of HMW-PAHs. For the uptake of substrates into microbial cells, the solubilization of these substrates is important. However, since HMW-PAHs are practically insoluble in water and thermodynamically stable, they are more resistant to microbial degradation. It was not until 1988, when Heitkamp *et al.* (1988) first isolated a *Mycobacterium* sp. (later designated as *Mycobacterium vanbaalenii* strain PYR-1) (Khan *et al.* 2002) with the potential to degrade pyrene, that the research on HMW-PAH biodegradation was accelerated. *Mycobacterium vanbaalenii* strain PYR-1 mineralized pyrene to a great extent (48% of added radiolabelled pyrene), as well as various other LMW- and HMW-PAHs including naphthalene, phenanthrene, fluoranthene, 3-methylcholanthrene, 1-

nitropyrene, and 6-nitrochrysene. Since this first report, a number of researchers have identified and reviewed bacteria capable of utilizing HMW-PAHs (Dean-Ross and Cerniglia 1996; Grosser *et al.* 1991; Kanaly and Harayama 2000; Rehmann *et al.* 1998; Walter *et al.* 1991). Most of the isolates capable of HMW-PAH degradation are nocardioform bacteria such as *Rhodococcus*, *Nocardia*, and *Mycobacterium* species, but some *Pseudomonas* species such as *Pseudomonas cepacia* (Juhasz *et al.* 1996) (reclassified as *Stenotrophomonas maltophilia* strain VUN 10,003 (Juhasz *et al.* 2000) were also isolated. Mueller *et al.* (1997) conducted a study on the diversity of PAH-degrading bacteria from various geographical regions and concluded that the degradation of certain PAHs is associated with members of certain taxa, regardless of the geographical origins from where the bacteria were isolated.

1.2.2. Mechanisms of PAH catabolism

Mineralization of PAHs by bacteria can be distinguished into two basic pathway types. Complete mineralization of a PAH first involves incorporation of dioxygen in an aromatic ring. This reaction is catalyzed by a dioxygenase, leading to *cis*-dihydrodiol intermediates. The intermediates are further broken down into smaller substrates and finally mineralized to carbon dioxide and water. As a rule, bacteria are able to use the PAH as a sole source of carbon and energy in complete mineralization.

Incomplete mineralization is significantly different from complete mineralization since PAHs are not mineralized to carbon dioxide and water. Instead, phenolic and carboxylic derivatives are accumulated as dead-end products at an early stage after initial hydroxylation. Bacteria are often unable to utilize the PAH for growth (Hinchel *et al.* 1994).

1.2.2.1. Biochemical pathway of LMW-PAH catabolism

The biochemical pathway of PAH catabolism has been extensively studied on LMW-PAHs because of their higher aqueous solubility and the ease of isolation and genetic manipulation of microorganisms capable of degrading LMW-PAHs (Cerniglia 1992). Since the first report of a biochemical pathway for naphthalene oxidation in 1964

(Davies and Evans 1964), the complete biochemical pathway, and genetic and enzymatic mechanisms involved in naphthalene catabolism have been elucidated (Figure 1.1) (Kurkela *et al.* 1988; Simon *et al.* 1993; Takizawa *et al.* 1999). The pathway for the degradation of naphthalene to salicylate is called the upper pathway and the pathway for the degradation of salicylate to central metabolites is called the lower pathway.

The phenanthrene catabolic pathway has also been identified (Kim and Zylstra 1999; Kiyohara *et al.* 1994) (Figure 1.2). For phenanthrene, there are two lower pathways for the transformation of phenanthrene into central metabolites. After phenanthrene is transformed to 1-hydroxy-2-naphthoate, it can either be converted to 1,2-dihyroxynaphthalene and enter a salicylate pathway as for naphthalene degradation, or be oxidized to *trans*-2'-carboxybenzalpyruvate and enter the phthalate pathway (Figure 1.2). The transformation of PAH substrates into central metabolites via the phthalate pathway has often been seen in the microbial degradation of PAH substrates with an increasing number of rings (Iwabuchi and Harayama 1998; Kelly *et al.* 1993; Vila *et al.* 2001).

Table 1.4. Polycyclic aromatic hydrocarbon (PAH)-degrading bacteria

Organisms	References
Naphthalene	
Acinetobacter calcoaceticus, *Alcaligenes denitrificans*, *Mycobacterium* sp. *Pseudomonas* sp. *P. putida*, *P. fluorescens*, *Sp paucimobilis*, *Brevundimonas vesicularis*, *Burkholderia cepacia*, *Comamonas testosteroni*, *Rhodococcus* sp., *Corynebacterium renale*, *Moraxella* sp., *Streptomyces* sp., *B. cereus*, *P.marginalis*, *P. stutzeri*, *P. saccharophilia*, *Neptunomonas naphthovorans*, *Cycloclasticus* sp.	Ryu *et al.* (1989), Weissenfels *et al.* (1990, 1991), Kelly *et al.* (1991), Dunn and Gunsalus (1973), Davies and Evans (1964), Foght and Westlake (1988), Jeffrey *et al.* (1975), Mueller *et al.* (1990), Kuhm *et al.* (1991), Walter *et al.* (1991), Dua and Meera (1981), Tagger *et al.* (1990), Garcia - Valdes *et al.* (1988), Trower *et al.* (1988), Grund *et al.* (1992), Barnsley (1975), Barnsley (1983), Yang *et al.* (1994), Burd and Ward (1996), Allen *et al.* (1997), Stringfellow and Aitken (1995), Filonov *et al.* (1999), Hedlund *et al.* (1999), Geiselbrecht *et al.* (1998), Foght and Westlake (1996), Goyal and Zylstra (1996)
Anthracene	
Beijernickia sp., *Mycobacterium* sp., *P. putida*, *Sphingomonas. paucimobilis*, *Rhodococcus* sp., *Flavobacterium* sp., *Arthrobacter* sp., *P. marginalis*,	Colla *et al.* (1959), Akhtar *et al.* (1975), Jerina *et al.* (1976), Evans *et al.* (1965), Ellis *et al.* (1991), Weissenfels *et al.* (1991), Foght and Westlake (1988), Walter *et al.* (1991),

Cycloclasticus sp., *P. fluorescens*, *Sp. yanoikuyae*, *Acinetobacter calcoaceticus*, *Gordona* sp., *Sphingomonas* sp., *C. testosteroni*, *Cycloclasticus pugetii Bu. cepacia*,	Mueller *et al.* (1990), Savino and Lollini (1977), Tongpim and Pickard (1996), Burd and Ward (1996), Geiselbrech *et al.* (1998), Foght and Westlake (1996), Kim *et al.* (1997), Lal and Khanna (1996), Mahro *et al.* (1995), Goyal and Zyltra (1996), Dyksterhouse *et al.* (1995), Allen *et al.* (1999)
Phenanthrene *Aeromonas* sp., *A. faecalis*, *A. denitrificans*, *Arthrobacter polchromogenes*, *Beijernickia* sp., *Micrococcus* sp., *Mycobacterium* sp., *P. putida*, *Sp. paucimobilis*, *Rhodococcus* sp., *Vibrio* sp., *Nocardia* sp., *Flavobacterium* sp., *Streptomyces* sp., *S. griseus*, *Acinetobacter* sp., *P. aeruginosa*, *P. stutzeri*, *P. saccharophila*, *Stenotrophomonas maltophilia*, *Cycloclasticus* sp., *P. fluorescens*, *Acinetobacter calcoaceticus*, *C. testosteroni*, *Acidovorax* al. *delafieldii*, *Gordana* sp., *Cycloclasticus pugetii*, *Sp. yanoikuyae*, *Agrobacterium* sp., *Bacillus* sp., *Burkholderia* sp., *Sphingomonas* sp., *Pseudomonas* sp., *Rhodotorula glutinis*, *Nocardioides* sp., *Flavobacterium gondwanense*, *Halomonas meridiana*	Kiyohara *et al.* (1976, 1982, 1990), Weissenfels *et al.* (1990, 1991), Keuth and Rehm (1991), Jerina *et al.* (1976), Colla *et al.* (1959), West *et al.* (1984), Kiyohara and Nagao (1978), Heitkamp and Cerniglia (1988), Guerin and Jones (1988, 1989), Treccani *et al.* (1954), Evans *et al.* (1965), Foght and Westlake (1988), Mueller *et al.* (1990), Sutherland *et al.* (1990), Ghosh and Mishra (1983), Savino and Lollini (1977), Trower *et al.* (1988), Barnsley (1983), Yang *et al.* (1994), Kohler *et al.* (1994), Stringfellow and Aitken (1995), Boonchan (1998), Juhasz (1998), Geiselbrecht *et al.* (1998), Foght and Westlake (1996), Kastner *et al.* (1998), Lal and Khanna (1996), Shuttleworth and Cerniglia (1996), Mahro *et al.* (1995), Goyal and Zylstra (1996), Dyksterhouse *et al.* (1995), Allen *et al.* (1999), Aitken *et al.* (1998), Romero *et al.* (1998), Iwabuchi *et al.* (1998), Churchill *et al.* (1999), Juhasz (1991)
Fluoranthene *A. denitrificans*, *Mycobacterium* sp., *P. putida*, *Sp. paucimobilis*, *Bu. cepacia*, *Pseudomonas* sp., *Strenotrophomonas maltophilia*, *Acinetobacter calcoaceticus*, *Acidovorax delafieldii*, *Gordona* sp., *Sphingomonas* sp., *P. saccharophilia*, *Pasteurella* sp.	Kelly and Cerniglia (1991), Walter *et al.* (1991), Weissenfels *et al.* *Rhodococcus* sp. (1991), Foght and Westlake (1988), Barnsley (1975), Mueller *et al.* (1990), Ye *et al.* (1996), Kelly *et al.* (1993), Boonchan (1998), Juhasz (1998), Lal and Khanna (1996), Shuttleworth and Cerniglia (1996), Mahro *et al.* (1995), Churchill *et al.* (1999), Chen and Aitken (1999)
Pyrene *A. denitrificans*, *Mycobacterium* sp., *Rhodococcus* sp., *Sp. paucimobilis*, *Strenotrophomonas maltophilia*, *Acinetobacter calcoaceticus*, *Gordona* sp., *Sphingomonas* sp., *P. putida*, *Bu. cepacia*, *P. saccharophilia*	Heitkamp *et al.* (1988), Walter *et al.* (1991), Weissenfels *et al.* (1991), Grosser *et al.* (1991), Schneider *et al.* (1996), Ye *et al.* (1996), Boonchan (1998), Juhasz (1998), Lal and Khanna (1996), Mahro *et al.* (1995), McNally *et al.*(1999), Jimemez and Bartha, Churchill *et al.* (1999),Chen and Aitken (1999)

Adapted from Juhasz and Naidu (2000)

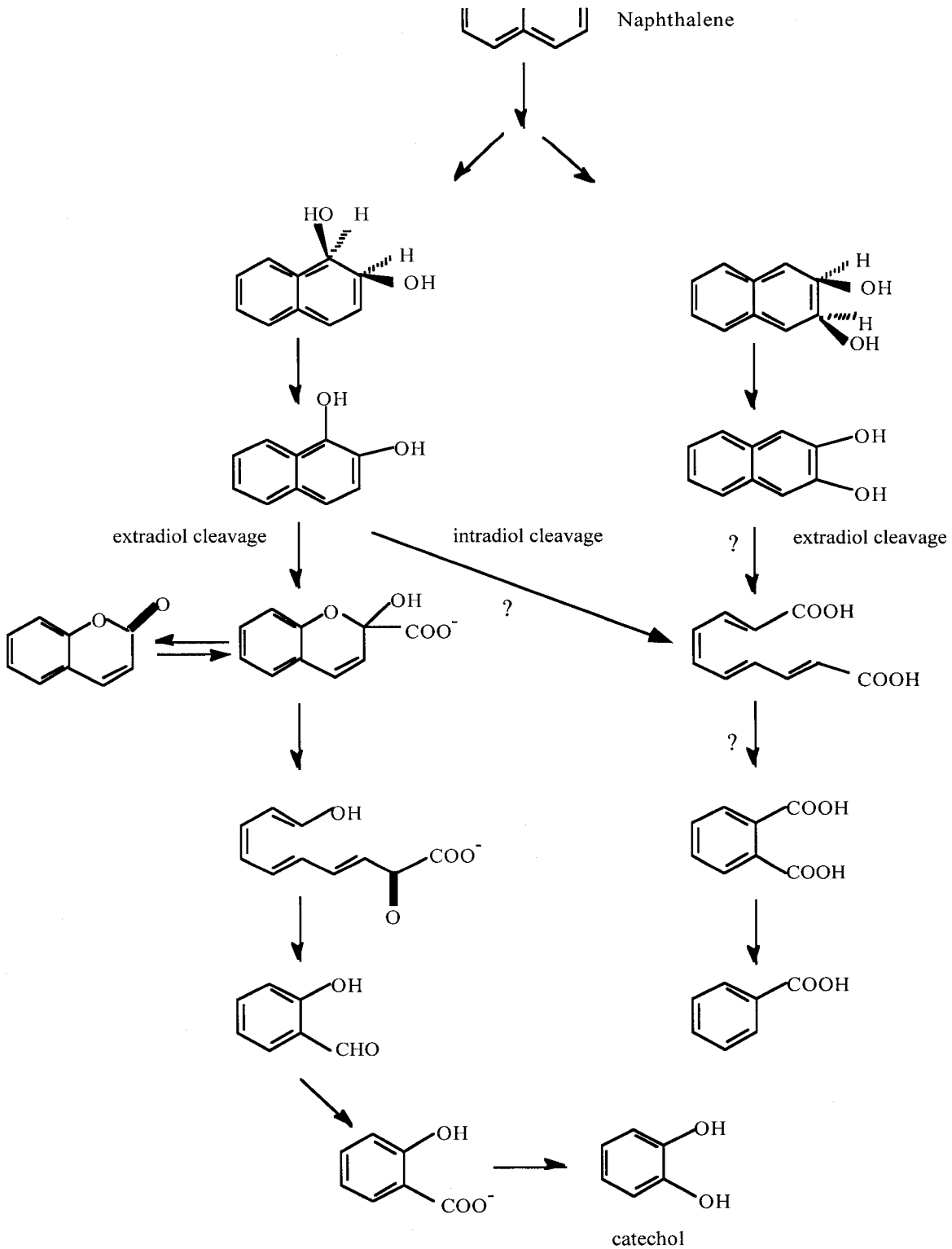


Figure 1.1. Naphthalene degradation pathway by mesophilic and thermophilic microorganisms (Annweiler et al. 2000).

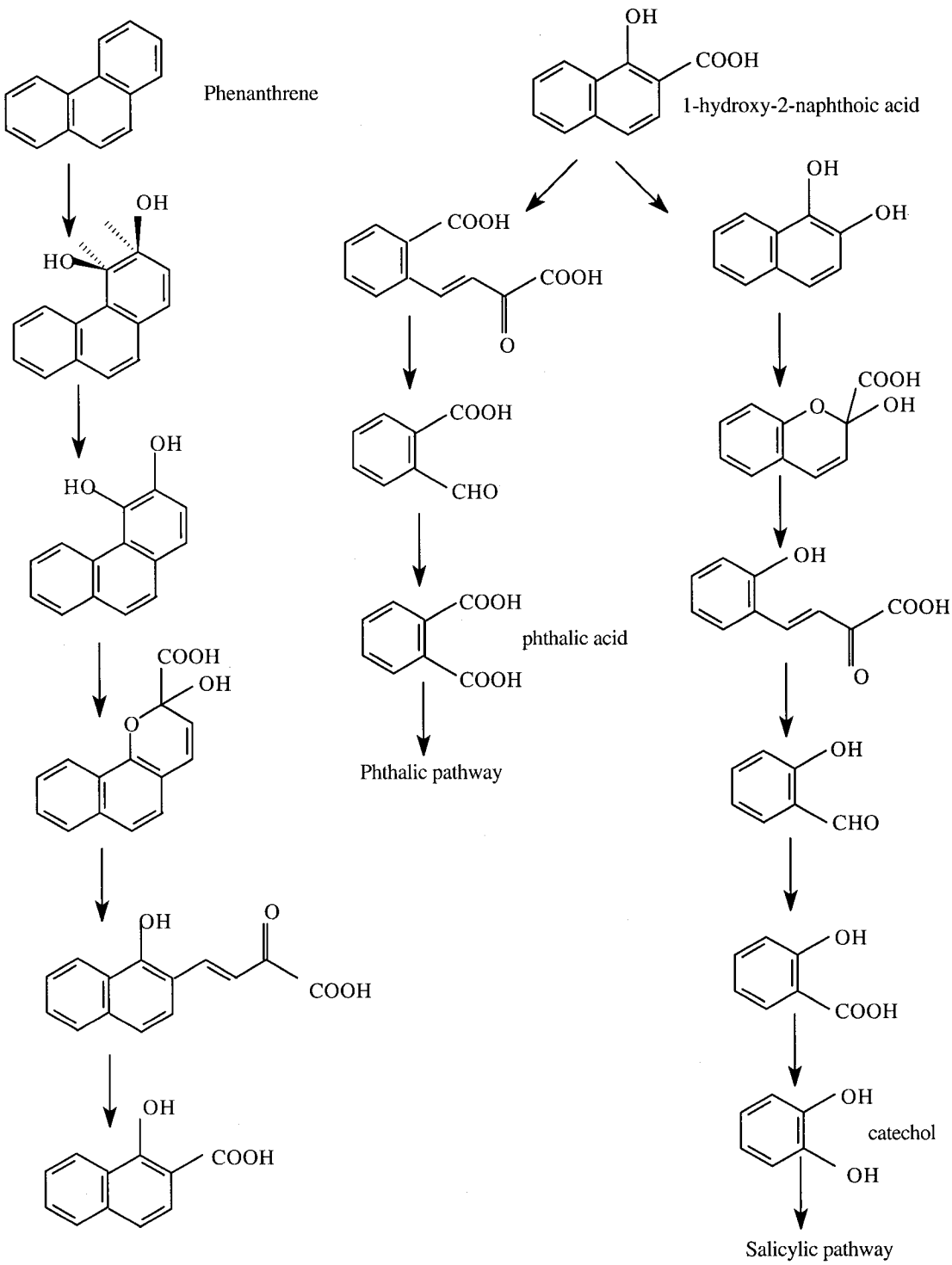


Figure 1.2. One of phenanthrene degradation pathways through dioxygenation of phenanthrene by bacteria. (Iwabuchi and Harayama 1997).

1.2.2.2. Bacterial aromatic ring-hydroxylating dioxygenases

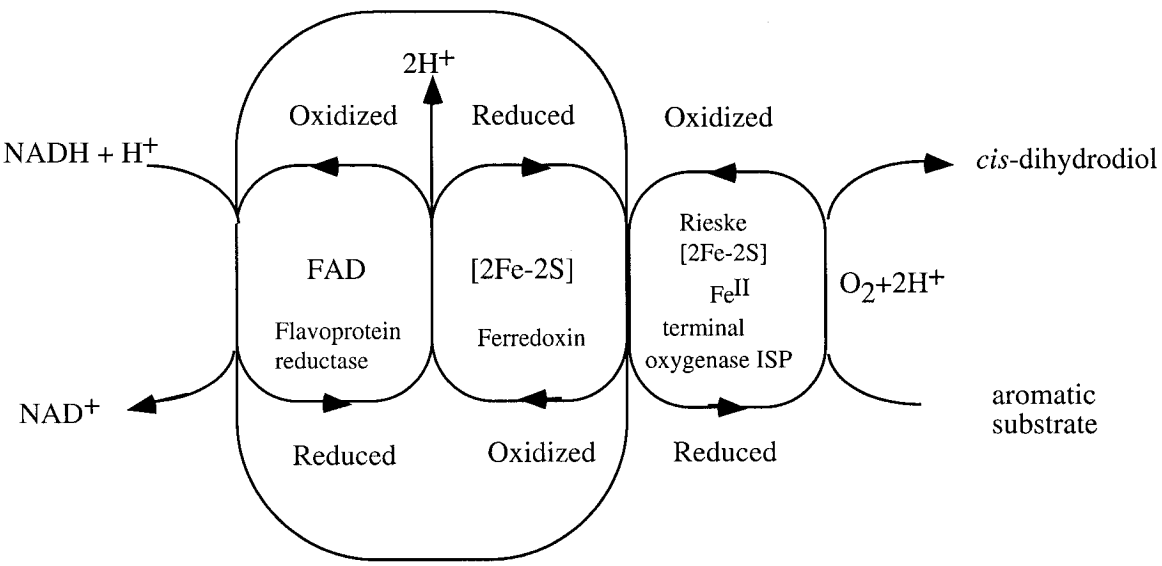
Initial catabolism of PAHs in bacteria involves the incorporation of both atoms of molecular oxygen into an aromatic ring to form a *cis*-dihydroxy-dihydrodiol. This reaction is catalyzed by a multicomponent enzyme system. A typical arrangement of the system consists of a short electron-transport chain composed of a NAD-dependent flavoprotein (FAD) reductase, a [2Fe-2S] ferredoxin, and a terminal oxygenase (iron-sulfur protein, ISP), consisting of an α (large) subunit and β (small) subunit (Figure 1.3). Electrons from NADH are transferred to the flavoprotein, which passes single electrons to ferredoxin. The ferredoxin then transfers electrons to the iron-sulfur protein (ISP) which uses the electrons to catalyze the transformation of the substrate into *cis*-dihydroxy-dihyrodiol (Butler and Mason 1997). The best known example is the naphthalene dioxygenase (NDO) multicomponent enzyme system (Figure 1.4) in *Pseudomonas* species.

The terminal oxygenase is an iron-sulfur protein (ISP) that is composed of two dissimilar subunits. The ISPα subunit is divided into the Rieske domain which contains the [2Fe-2S] centre and the catalytic domain which contains the non-heme ferrous iron. The Rieske [2Fe-2S] centre, when reduced, transfers electrons to the mononuclear iron at the active site in the catalytic domain. The amino acid sequence of the Rieske [2Fe-2S] centre is conserved in all bacterial ring-hydroxylating dioxygenases. It has a consensus sequence of Cys-X_1-His-X_{16}-Cys-X_2-His (PROSITE accession number PS00570), where two cysteine (C) and two histidine (H) residues are fully conserved. The α subunit of the ISP is also responsible for determining substrate specificity. Site-directed mutagenesis assays by Parales *et al.* (2000) determined that an amino acid substitution at Phe-352 of the α subunit ISP in the naphthalene dioxygenase (NDO) system of *Pseudomonas* sp. strain NCIB9816-4 altered the NDO substrate specificity. The specific substitution caused the alteration of the site of oxidation of substrates and of the stereochemistry of metabolites (Parales *et al.* 2000).

Some literature has suggested that the β subunit ISP may contribute to substrate specificity. However, a recent study indicated that the β subunit ISP has no cofactors and its main role is most likely structural. This hypothesis is supported by the fact that some

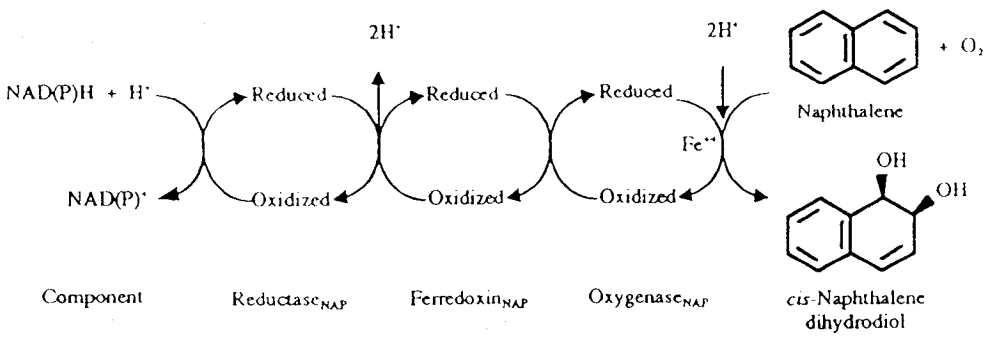
atoms of the β subunit ISP are positioned very close to the iron at the active site and the Rieske centre of the α subunit ISP (Kauppi *et al.* 1998).

The genes responsible for PAH degradation have been extensively investigated in naphthalene catabolism and the genes have been cloned from *Pseudomonas* strains because of the ease of genetic manipulation in *Escherichia coli.* The best characterized naphthalene dioxygenase genes are the *nah* and *ndo* genes from two naphthalene degrading organisms, *Pseudomonas putida* strain G7 and *Pseudomonas putida* strain NCIB9816-4, respectively (Simon *et al.* 1993). The *nah* and *ndo* genes are highly conserved among various naphthalene-degrading bacteria and are 93% homologous to each other at the nucleotide level. Other genes such as the *pah* genes from a phenanthrene-degrading organism, *Pseudomonas putida* OUS82 (Takizawa *et al.* 1994) also show a high degree of homology to the *nah* genes. Many bacteria isolated from diverse geological



Adapted from Butler and Mason. 1997.

Figure 1.3. Biochemical organization of the multicomponent ring-hydroxylating dioxygenase



Adapted from Parales *et al.* 2000.

Figure 1.4. Multicomponent enzyme system of naphthalene dioxygenase

degrading naphthalene and phenanthrene seem to possess the *nah*-like genes. They have been used as models for the detection of PAH-degrading organisms from contaminated sites, and for determining the presence of PAH degradation potential in microbial populations (Fleming *et al.* 1993; Herrick *et al.* 1993; Lloyd-Jones *et al.* 1999; Wilson *et al.* 1999).

Because of the wide spread occurrence of these genes in bacteria from various locations, it was speculated that the genetic diversity for the degradation of PAHs was limited. However, recent studies have shown significant diversity among the genes involved in the catabolism of PAHs (Goyal and Zylstra 1996; Iwabuchi and Harayama 1997; Laurie and Lloyd-Jones, 1999). Laurie and Lloyd-Jones (1999) reported that only 45% of bacterial strains isolated with the naphthalene-degrading phenotype were successfully screened with the *nah* gene probes. In the case of isolates with phenanthrene-degradation potential, which are also able to degrade naphthalene, only 15% of the isolates were effectively screened with the same probes (Laurie and Lloyd-Jones 1999). It is now accepted that screening with one highly conserved group of genes such as the *nah* or *ndo* genes may not represent the true diversity of genes involved in PAH degradation.

The PAH catabolic genes in *Comamonas testosteroni* GZ39, the *phn* genes in *Burkholderia* sp. strain RP007, and the *phd* genes in *Nocardioides* sp. strain KP7 are all isofunctional genes with low homology to each other and to the well known *nah* genes in *Pseudomonas putida* sp. strain G7. All these genes are significantly different in sequence and gene order from the *nah* and *nah*-like, i.e., *pah*, genes. Southern hybridization using the *nah* and *pah* gene probes on *Burkholderia* sp. strain RP007 revealed that there was no homology between the *phn* and the *nah* and *pah* genes. The same result was shown with the genes in *Comamonas testosteroni* GZ39 (Zylstra *et al.* 1997)

Recently, a gene encoding the initial hydroxylation of pyrene, *nid*A, has been characterized and sequenced from *Mycobacterium* sp. strain PYR-1 (Khan *et al.* 2001). A phylogenetic tree for the α subunit ISP of various dioxygenases revealed that the pyrene dioxygenase, NidA, does not cluster with the classic Nah-like dioxygenases. Instead, it clusters with the α subunit ISP of a newly described dioxygenase from *Rhodococcus* sp. strain I24 and *Nocardioides* sp. KP7. In the phylogenetic tree, dioxygenases found in Gram-negative bacteria clustered together and dioxygenases found in Gram-positive bacteria clustered together. This may suggest that Gram-positive bacteria have evolved different mechanisms for the utilization of PAHs as compared to the degradation mechanism of Gram-negative bacteria.

1.3 Goals of this Study

The primary goal of this study was to characterize a pyrene degrading bacterium, *Mycobacterium* sp. strain S65 at molecular level and as a potential bioremediation agent in PAH contaminated soils.

To understand pyrene degradation at molecular level, cloning of pyrene catabolic genes has been performed using strain S65 and subsequently sequencing of the genes to identify their potential activities. This study was of interest because only one pyrene initial hydroxylation gene was previously identified and it is important to characterize the genetic mechanism of pyrene degradation to understand how bacteria approach to utilize such a pollutant as carbon and energy sources.

Bioaugmentation of a PAH contaminated soil collected from a fire fighter training site was performed using strain S65 as a bioremediation agent. Various conditions were tested to determine an optimum condition for S65 to remediate PAHs from the soil. The survival of strain S65 in the indigenous microbial community was determined using a combination of denaturing gradient gel electrophoresis (DGGE)- hybridisation technique. This study was applied to understand the behaviour of strain S65 among other microorganisms and the efficiency of strain S65 as a bioremediation agent.

Connecting Text

As described in Literature Review, the adverse effects of PAHs, especially HMW-PAHs in the environment and to humans are of concern. Because of the difficulty isolating HMW-PAH degrading bacteria our knowledge in genetic mechanisms of HMW-PAH biodegradation has been limited. In Chapter 2, an extensive study of Mycobacterium sp. strain S65 was carried out on a genetic mechanism of pyrene (one of HMW-PAHs) degradation. Michiei Sho designed and performed all of the experiments, accompanying analyses and writing of the manuscript. Drs. Charles W. Greer and Chantal Hamel provided valuable suggestions to improve the study.

Chapter 2. Molecular Analysis of Pyrene Degradation by *Mycobacterium* sp. strain S65.

This work presented in this chapter is being compiled into a manuscript for potential publication. The title of the manuscript is "Molecular Analysis of Pyrene Degradation by *Mycobacterium* sp. Strain S65." I was the author and performed the experimental work. The co-authors participated in this design of the experiments, in analysis of the data and revision to the manuscript are Drs. Charles W. Greer and Chantal Hamel.

2.1. Abstract

A pyrene degrading bacterium, identified as *Mycobacterium* sp. strain S65, was isolated from a jet-fuel contaminated site in Sept-Iles, northern Quebec, Canada. S65 utilized pyrene (60% mineralization), phenanthrene (60% mineralization), and fluoranthene (20% mineralization) as sole carbon and energy sources, but did not degrade naphthalene, anthracene, and fluorene. Pyrene mineralization was enhanced by adding benz[a]anthracene, benzo[a]pyrene, or phenanthrene as cosubstrates.

Southern hybridization using a pyrene dioxygenase, *nidA* gene probe, characterized in another pyrene-degrading bacterium, *Mycobacterium* sp. strain PYR-1, indicated that *nidA* homologues were found in more than one location in S65. Two positive clones were obtained and sequenced. Five and seven open reading frames (orfs) were found in clone 1 (*nid* locus) and in clone 2 (*pdo* locus), respectively. Based on a homology search, two *orf*s in each locus were predicted to encode bacterial ring-hydroxylating dioxygenase genes. Only one *orf* in clone 1 showed 99.5% nucleotide similarity to the *nidA* gene, while the corresponding orf in clone 2 was only 89% homologous. The second large subunit dioxygenase had no nucleotide similarity to any known genes, but showed >60% similarity to a hypothetical dioxygenase in a dioxin degrading bacterium, *Sphingomonas* sp. strain RW1 at the amino acid level. Orfs encoding consensus regions for a short-chain alcohol dehydrogenase and a zinc-containing alcohol dehydrogenase were detected downstream of the *nidA* homologues in both loci.

Sequence comparisons of the two loci revealed that the corresponding orfs had consistent similarity (89%), suggesting that one locus might have originated from the other. This was strongly supported by the presence of a transposase gene in the *pdo* locus.

2.2. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous organic pollutants in nature. PAHs are mainly produced as a result of incomplete combustion of organic materials such as fossil fuels, by-products of mobile exhaust, cigarette smoke, and during cooking. PAHs are considered as health hazards because some of them possess mutagenic, genotoxic, and/or carcinogenic activities. The degree of toxicity of PAHs increases proportionally to the number of aromatic rings (Binkova *et al.* 2000; Marston *et al.* 2001). For example, 3-ring PAHs such as anthracene and phenanthrene do not have any activities in bacterial mutagenicity, carcinogenicity, and DNA adduct formation. On the other hand, the 4-ring PAH, pyrene, showed mutagenic activity in bacteria, and the 5-ring PAH, benzo[a]pyrene, showed very high carcinogenic activity in mouse skin (Ingram *et al.* 2000).

The microbial degradation of pyrene has been elucidated with the increasing number of pyrene-degrading bacteria isolated in recent years. A number of studies have reported successful degradation of high-molecular-weight (HMW)-PAHs by different bacteria (Churchill *et al.* 1999; Dean-Ross and Cerniglia 1996; Heitkamp and Cerniglia 1988; Ho *et al.* 2000; Juhasz *et al.* 2000; Rehmann *et al.* 1998; Walter *et al.* 1991). Most HMW-PAH degrading bacteria have also shown the capacity for utilizing other PAHs. *Mycobacterium vanbaalenii* strain PYR-1 could mineralize naphthalene (59.9%), phenanthrene (50.9%), and fluoranthene (89.7%) in addition to pyrene (63%) in 2 weeks in the presence of low concentrations of organic nutrients. *Mycobacterium* sp. strain CH1 has been reported to degrade PAHs including phenanthrene, fluoranthene, and pyrene, as well as a wide range of alkanes as sole carbon and energy sources (Churchill *et al.* 1999). When a high cell density of *Stenotrophomonas maltophilia* strain VUN10,003, one of a few Gram-negative HMW-PAH degrading bacteria, was introduced in media containing PAHs, fluoranthene, pyrene, benz[a]anthracene, benzo[a]pyrene, dibenz[a,h]anthracene, and coronene disappeared from the media at 45%, 98%, 26%, 23%, 22%, and 55% of the introduced concentration, respectively (Juhasz *et al.* 2000).

Enzymes and pathways involved in PAH catabolism have been extensively studied and reviewed in the past several decades. However, our understanding of the

bacterial degradation of PAHs mainly came from studies done on the bacterial degradation of low-molecular-weight (LMW) PAHs (Kim and Zylstra 1999; Kiyohara *et al.* 1994; Laurie and Lloyd-Jones 1999; Simon *et al.* 1993; Stillwell *et al.* 1995). This was due to the ease of isolating microorganisms capable of degrading LMW-PAHs and the high solubility of LMW-PAHs, which has made it easier to identify intermediate metabolites. Genetic characterization was also advanced for LMW-PAH degradation because most of the bacterial isolates were Gram-negative, making the manipulation and expression of genes in *E. coli* possible.

The enzymes and genes involved in HMW-PAHs degradation have not yet been fully elucidated. This has been made difficult due to the low aqueous solubility of HMW-PAHs and only a few organisms have been isolated as potential HMW-PAH degraders, the majority being Gram-positive actinomycetes. Recently, Khan *et al.* (2001) reported the successful cloning of an initial pyrene dioxygenase gene, *nidA*, from a pyrene degrading bacterium, *Mycobacterium vanbaalenii* strain PYR-1. The complete sequence of the gene revealed that it was distantly related to classic bacterial ring-hydroxylating genes such as the *nahAc*, *ndoB*, and *phnAc* genes from *Pseudomonas* and *Burkholderia* species. Instead, it clustered with high homology to the newly described dioxygenase genes, *nidA* and *phdA* from *Rhodococcus* sp. strain I24 and *Nocardioides* sp. strain KP7, respectively. The catabolic pathway of pyrene degradation has also been elucidated by a number of scientists (Heitkamp *et al.* 1988; Kazunga and Aitken 2000; Rehmann *et al.* 1998; Vila *et al.* 2001). Studies on metabolites of pyrene degradation have proposed that the end product of pyrene catabolism was phthallic acid and this finding was consistent with most of the bacterial metabolites of PAHs with more than 3 rings.

Mycobacterium sp. strain S65 was isolated in our laboratory from a site contaminated with jet-fuel in northern Quebec, Canada. The main objectives of this study were to evaluate the ability of this strain as a pyrene (and other PAHs) degrader and to understand the genetic mechanism of pyrene degradation.

2.3. Materials and Methods

2.3.1.Isolation of bacterial strain and culture conditions

The bacterial strain, referred to as S65, was isolated by Jamshid Jazestani from a soil contaminated with jet fuel in Sept-Iles, northern Quebec. Five g of the soil was serially diluted in 3 volumes of 0.1% sodium pyrophosphate buffer, pH 7.0, and plated in triplicate on low nutrient agar plates, YTS250 (0.25g each of yeast extract, tryptone, and starch per litre, pH 7.0) plates, overlaid with phenanthrene.(Greer *et al.* 1993) A colony producing a clearing zone on the plates was selected and purified for further characterization. The colony was routinely subcultured at 30°C on YTS1000 plates containing 1.0 g each of yeast extract, tryptone, and starch per litre of distilled water.

2.3.2. Biochemical and physiological analyses

Micromorphological properties of S65 were determined by phase-contrast microscopy. Gram staining, catalase and oxidase tests, and acid-alcohol fastness (Ziehl-Neelsen method) were tested on S65 as previously described (Murray *et al.* 1994).

To determine the optimum growth temperature for S65, 500-ml side-arm flasks containing 60-ml YTS1000 broth, were inoculated with 1 ml of mid-log phase culture and incubated at 10°C, 20°C, 25°C, 30°C, and 35°C. Cultures were set up in triplicate for each of the temperatures and the OD at 600 nm was measured until the cultures reached stationary phase.

The carbon utilization pattern of S65 was determined using Biolog GP2 Microplates (Biolog, Hayward, CA). The Biolog GP2 Microplate was developed to identify Gram-positive bacteria by determining the utilization pattern of 95 different carbon sources (biochemical characterization). In this study, the technique was applied to determine the functional diversity of the bacterial strain, and not to identify it. A culture was grown in a YTS1000 broth to mid-log phase and harvested. The culture was washed with 0.85% (v/v) NaCl solution twice and an aliquot was added to each well containing the different substrates and incubated at 30°C. The OD_{590} was measured after 24 hr incubation. Positive growth was calculated based on an OD_{590} of 1.4 times the reference well (H_20).

2.3.3. Bacterial genomic DNA extraction

DNA was extracted by a modified version of Ausubel's method (Ausubel *et al.* 1998): a culture grown until saturation was harvested at 4,500 X g at 25°C for 10 min. The cell pellet was resuspended in TE (10 mM Tris, 0.1 mM EDTA [pH 8.0]) buffer; an aliquot of the cell suspension was added to a 2-ml screw-cap tube containing 0.5 g of sterile 0.1mm-diameter zirconium-silica beads (BioSpec Products, Bartlesville, OK). The suspension was homogenized using a Mini Bead-Beater 8 (BioSpec Products) for two minutes. The tube was then centrifuged at 16,000 X g for 5 minutes. The supernatant was transferred to a new tube containing 20% SDS and 20 mg/ml proteinase K and incubated at 37°C for 1 hour. Denaturation of protein and polysaccharide complexes was performed by the addition of 80 µl hexadecyltrimethylammonium - bromide and 100 µl 5 M NaCl (CTAB/NaCl), followed by incubation at 65°C for 10 min. Polysaccharides and other contaminating macromolecules were separated from the genomic DNA twice with 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1), and twice with chloroform/isoamyl alcohol (24:1). The DNA preparation was precipitated with 1 volume of isopropanol at –20°C overnight. The tube containing the DNA precipitate was centrifuged at 16,000 X g for 20 min at 4°C. The DNA pellet was washed with 70% ethanol prior to drying under vacuum. The DNA pellet was dissolved in TE buffer.

2.3.4. Identification of Strain S65

The genus of strain S65 was identified by direct sequencing of 16S rDNA. The polymerase chain reaction (PCR) was performed to amplify the entire 16S rDNA using universal sequencing primers with the following sequences: 5'–GAGTTTGATCCTGGCTACG–3' (11–29, *Escherichia. coli* numbering) as a forward primer and 5'–AGAAAGGAGGTGATCCAGCC–3' (1525–1544, *E. coli* numbering) as a reverse primer (Dorsch and Stackebrandt 1992). The PCR reaction consisted of 0.1 - 1 ng/µl of genomic DNA, 0.4 pmol of each primer, 200 µM of each deoxyribonucleoside triphosphate, and 4 mM $MgCl_e$. The PCR reaction conditions were as follows: the DNA was denatured at 96°C for 2 min. The temperature was decreased to 80°C while adding 1.25 U *Taq* DNA polymerase, in 5 µl of 10X PCR buffer (10 mM Tris-HCl [pH 9.0], 50

mM KCl, 1.5 mM $MgCl_2$) (Amersham Pharmacia Biotech, Piscataway, NJ). The PCR cycling parameters were: 1 min of 94°C, 1 min of 62°C, and 1 min of 72°C for a total of 30 cycles. The PCR amplicon was purified using the QIAQuick PCR purification kit (Qiagen, Mississauga, ON) to remove residual enzymes and primers, which interfere with the subsequent sequencing reaction. The sequencing reaction contained 15 ng of the PCR amplified DNA, 10 pmol of appropriate sequencing primer, 4 µl ABI Prism dRhodamine reaction mix (Perkin Elmer Applied Biosystems, Warrington, UK). Deionized H_2O was added to a final volume of 10 µl. The sequencing conditions were as follows: 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min for a total of 25 cycles. Sequencing was performed using the ABI Prism 377 automated fluorescence sequencer (Perkin Elmer Applied Biosystems) according to the manufacturer's instructions. The sequence was submitted to the GeneBank database to search for homology with sequences of other bacteria by using the Blast alignment tool (Altschul *et al.* 1997).

2.3.5. Mineralization of PAHs

The mineralization assay was prepared in triplicate serum bottles each containing mineral salts medium (MSM) (Whyte *et al.* 1996) at 30°C with slow agitation. An 800 µl aliquot of the culture with an OD_{600} of 0.1 was added to make a final volume of 20 ml. A heat killed culture was used as a negative control. The control culture was autoclaved twice with the second autoclaving being performed one day after the first autoclaving. Five ml test tubes containing 1 ml of 0.5N KOH as a CO_2 trap were placed inside each serum bottle. The ^{14}C-labelled substrates (Sigma, Oakville, ON) used in this assay were [1,2,3,4,4a,9a-^{14}C]anthracene (specific activity, 11.2 mC/mmol), [9-^{14}C]fluorene (specific activity, 14.55 mCi/mmol), [3-^{14}C]fluoranthene (specfic activity, 45 mCi/mmol), [1-^{14}C]naphthalene (specific activity, 8.1 mCi/mmol), [9-^{14}C]phenanthrene (specific activity, 46.9 mCi/mmol), and [4,5,9,10-^{14}C]pyrene (specific activity, 58.7 mCi/mmol); 100,000 dpm of each ^{14}C-labeled substrate was added to each serum bottle. The serum bottles were sealed with Teflon coated rubber stoppers and aluminium crimps. The KOH was routinely removed using a needle attached to a syringe, and another 1 ml of fresh KOH was added to the tube to clean the wall of the tube and pooled. The pooled KOH (2 ml)

was mixed with 18 ml of scintillation cocktail (Fisher Sci., Whitby ON) and the rate of the mineralization was measured by liquid scintillation spectrometry.

Another assay to study pyrene mineralization was carried out in the presence of other PAHs (phenanthrene, fluoranthene, benzo[a]pyrene, and benz[a]anthracene) as described above. The only radiolabelled substrate was pyrene. The substrates were added at a concentration of 1 mg/L, either individually or as a mixture to determine the impact of the different substrates on pyrene degradation. The effect of culture conditions on pyrene mineralization was also studied. S65 was either pre-grown in YTS1000 broth as a non-induced culture, or in MSM broth supplemented with 10 mg/L pyrene as an induced culture.

2.3.6. Screening for large plasmids

The strain was examined for the presence of large plasmids. A culture in YTS1000 broth was grown until saturation and harvested at 4°C. Lysis buffer (50mM glucose, 25mM Tris, pH8.0, and 10mM EDTA) containing 10 mg/mL lysozyme was added and the suspension was incubated at 37°C for 30 min. An aliquot of the suspension was transferred to a new tube and lysis was completed with a solution containing 0.2N NaOH and 4% SDS on ice for 10 min. High molecular weight genomic DNA and RNA were precipitated by adding 5M potassium acetate, pH 4.8, while plasmid DNA remained in the supernatant. Plasmid DNA was further purified from other cellular components using phenol/chloroform/isoamyl alcohol (25:24:1) extraction. The plasmid DNA was precipitated with 1 volume of isopropanol at –20°C overnight. The tube containing plasmid DNA was centrifuged and washed with 70% ethanol. The plasmid preparation was air dried and dissolved in TE buffer. An aliquot was loaded onto a 0.5% agarose gel in Tris-borate buffer and electrophoresed at 3V/cm overnight for verification. The gel was stained in ethidium bromide solution (1 mg/L) and visualized under UV light.

2.3.7. Cloning the pyrene catabolic genes

Genomic DNA was individually digested with *Bam*HI, *Eco*RI, *Eco*RV, *Hind*III, *Sac*I, *Sac*II, *Sal*I, *Spe*I, and *Xba*I (New England Biolabs, Mississauga, ON). The DNA

digests were fractionated on a 0.5% agarose gel in Tris-borate buffer overnight. Capillary transfer of the digested DNA onto a membrane was carried out using a Southern hybridization apparatus (ALTEC Labs, Boston, MA) as described in Sambrook *et al.* (1989). The gel was placed on a stone soaked in 20X SSC buffer (3M NaCl and 300 mM sodium citrate, pH 7.0). Buffer was drawn from the stone and passed through the gel. The membrane was placed on the top of the gel and the DNA was slowly eluted from the gel onto the membrane. Two pieces of 3MM Whatman paper, a stack of paper towels, and a weight (approximately 500 g) were applied on top of the membrane to give a tight bond between the gel and the membrane. The transfer was performed for 24 hr. After the transfer, DNA was cross-linked onto the membrane with UV and briefly rinsed with 5X SSC. The membrane was air dried and kept at room temperature until used.

A 500-bp PCR fragment of the naphthalene inducible dioxygenase (*nidA*) gene, found in *Mycobacterium* sp. strain PYR-1 (GenBank AF249301) (Khan *et al.* 2001), prepared using S65 genomic DNA, was used as a probe. The forward primer, 5'-ATCTTCGGGCGCGGCTGGGTGTTTCTCGG-3' and the reverse primer, 5'-AATTGTCGGCGGCTGTCTTCCAGTTCGC-3' were designed to include the Rieske iron-sulfur binding site signature sequence of the *nid*A gene. The gene was PCR amplified using a cycle of 96°C, 65°C, and 72°C, each for 1 min. for a total of 30 cycles. The PCR amplicon was labelled with a DIG labelling system according to the manufacturer's instructions (Roche Boehringer Mannheim, Laval, QC). An aliquot (200 ng) of the PCR amplicon was denatured in boiling water for 10 min., and incubated on ice right after the heat treatment. The denatured PCR amplicon was then mixed with 10X hexanucleotide mix, 10X dNTP mix, 3U Klenow fragment (NEB), and water to make a final volume of 20 µl. Labelling proceeded at 37°C overnight. The reaction was stopped by adding 0.8 µl of 0.5M EDTA, pH 8.0. The final volume of the reaction mix was adjusted to 50 µl with TE, pH 8.0. The DNA probe was precipitated with 1 µl glycogen (20 mg/ml), 5.1 µl 3M sodium acetate, pH 5.2, and 140 µl of ethanol at –80°C for 30 min. The DNA probe was dissolved in 50 µl TE containing 0.1% SDS and stored at –20°C until used.

Southern hybridization was performed as follows: the membrane was soaked in a pre-hybridization solution (5X SSC, 0.1% N-lauroyl sarcosine, 0.02% SDS, and 1%

blocking reagent (Roche) at 65°C for 1 hr with slow agitation. The pre-hybridization solution was discarded and hybridization was carried out at 65°C for 16 hr after adding a fresh hybridization solution containing a DIG-labelled probe. After hybridization, the membrane was washed twice with 2X SSC at room temperature, and twice with 1X SSC. The washing process was completed by transferring the membrane into 0.5 X SSC and mixing at 65°C. Each washing step lasted 15 min. Hybridized fragments were detected using chemiluminescence according to the manufacturer's instruction (NEB). The membrane was stabilized in solution 1 (0.1M maleic acid and 0.15M NaCl, pH7.5) containing 0.3% Tween-20 (v/v) for 5 min. Antibodies (Anti-Digoxigenin Fab fragments, Roche) were added in solution 2 (1% blocking agent in solution 1) and the membrane was incubated for 30 min with low agitation. Free antibodies were removed by washing the membrane twice in solution 1 containing 0.3% Tween-20 for 15 min each time. The membrane was stabilized in solution 3 (10 mM Tris-Hcl, pH 9.5, 10 mM NaCl, and 1 mM $MgCl_2$) for 2 min with agitation and incubated in a CDP solution for 30 sec). The membrane was then exposed to X-ray film (X-Omat, Kodak, Rochester, NY) for 30 sec for the detection of hybridization signals. Two fragments with positive signals were further investigated by cloning and nucleotide sequencing.

The two size fractions (7 kb and 11 kb fragments of *Eco*RV digested genomic DNA) that hybridized to the probe were extracted from a low-melting agarose gel (Bio-Rad, Mississauga, ON). Briefly, pieces of the gel containing the fragments were cut out and transferred to microcentrifuge tubes and heated at 65°C for 10 min. An equal volume of phenol was added, vortexed, then centrifuged at 16,000 X g for 5 min. The aqueous phase was transferred to a new tube. DNA was extracted with phenol three times and precipitated with 300 mM NaCl and 2.5 vol of ethanol at –20°C for 2 hr or longer. The DNA was washed, dried and dissolved in TE, pH 8.0, as described for the genomic DNA preparation. The purified DNA fragments were separately ligated to pBluescriptIIKS. The vector was digested with an appropriate restriction enzyme and treated with calf intestinal alkaline phosphatase (CIP) (NEB) to dephosphorylate the 5' end of the vector to prevent self-annealing. Dephosphorylation was carried out at 37°C for 1 hr, then the reaction was stopped by adding 0.2M EGTA, pH7.5, at 65°C for 10 min. The vector was

extracted using phenol/chloroform/isoamyl alcohol extraction as previously described in this chapter.

DH10B competent cells were prepared using a modified version of the method developed by Simanis (Hanahan 1985). Briefly, several colonies of DH10B were inoculated into LB broth, at 37°C until an OD_{550} of 0.5 was obtained. The culture was then incubated on ice for 1 hr followed by centrifugation at 4,500 X g at 4°C for 10 min. The cell pellet was resuspended in RF1 solution (100 mM RbCl, 50 mM $MnCl_2 \bullet 4H_2O$, 35 mM potassium acetate, 10 m $CaCl_2 \bullet 2H_2O$, and 15% glycerol) at 1/3 of the original volume and incubated on ice for 1 hr. The supernatant was discarded after centrifugation and the cell pellet was resuspended in RF2 buffer (10 mM MOPS, 10 mM RbCl, 75 mM $CaCl_2 \bullet 2H_2O$, and 15% glycerol) at 1/12.5 of the original volume. The suspension was incubated on ice for 15 min. Aliquots were transferred to tubes and immediately placed in a dry ice/ethanol bath and stored at –80°C.

The ligation reaction contained 1 mM ATP, 40% PEG-8000, 1/10 volume of One-Phor-All buffer (Amarsham Pharmacia Biotech), 3U of T4 DNA ligase, DNA fragment and vector in a 3:1 molar ratio, and water to make up a final volume of 15 µl. The ligation mixture was incubated at 16°C overnight then chemically transformed into *E. coli* DH10B competent cells according to the protocols described in Sambrook *et al.* (1989). The transformed cells were plated on LB plates containing 100 µg/ml of ampicillin. Prior to plating, all the plates were overlaid with 50 µl of 2% X-Gal and 20 µl of 0.1M IPTG per plate for selection of positive clones by a blue-white assay.

White colonies on the plates were transferred to master plates (LB + Amp_{100}) and grown overnight. Colonies were lifted onto membranes (Roche) and treated in denaturation solution (0.5N NaOH-1.5M NaCl) for 15 min. After quickly drying on Whatman filter paper, the membranes were neutralized in a solution containing 1M Tris, pH 7.5 and 1.5M NaCl, for 15 min. The membranes were then soaked in 2X SSC for 10 min. DNA was fixed onto the membranes using the UV Stratalinker 1800 (Stratagene, La Jolla, CA). Hybridization and detection were carried out in the same manner as for Southern hybridization. Colonies corresponding to the hybridization signals were picked from the master plates and screened for the *nidA* gene by PCR. The clones with strong amplifications were selected and sequenced. Oligonucleotides designed from both the

KS, 5'-CGAGGTCGACGGTATCG-3', and SK, 5'-TCTAGAACTAGTGGATC-3', ends of the BluescriptKSII plasmid were used to sequence starting at the ends of the inserts. To complete the gaps, oligonucleotides were designed from the ends of assembled sequences. Sequencing was performed as described in the bacterial identification section, except the BigDye v.2 reaction mix was used instead of the dRhodamine reaction mix. Sequences were manually assembled and edited using Gap4 from the Staden Sequence Analysis software package. The assembled sequences were generated as a FASTA file and submitted to the GenBank database to search for homology with existing genes by using Blast alignment tool.

2.4. Results

2.4.1. Strain identification

A bacterial strain capable of utilizing phenanthrene and pyrene as sole carbon sources was obtained from an airport in Sept-Iles, northern Quebec, Canada. The strain was found to be Gram variable, catalase-positive, with a rod-coccus morphology, and produced creamy yellow circular colonies. Direct sequencing of PCR amplified 16S rDNA of this isolate revealed that this bacterium belonged to the genus *Mycobacterium*. A 1473 base-pair (bp) sequence (Appendix 1-1) was submitted to the GenBank database to search for homology with other bacteria. The result showed 99% similarity to *Mycobacterium* sp. strain T104/T103 and 98% to *Mycobacterium* sp. strain RJGII-135 (Appendix 1-2). *Mycobacterium* sp. strain T104/T103 is a toluene degrading bacterium isolated from rock surface biomass in freshwater contaminated with toluene (Tay *et al.* 1998). *Mycobacterium* sp. strain RJGII-135 was isolated from a former coal gasification plant in southern Illinois and has the capacity to degrade four- and/or five-ring PAHs (Govindaswami *et al.* 1995). The sequence identity to the well-known pyrene degrading bacterium, *Mycobacterium* sp. strain PYR-1 (later reclassified as *Mycobacterium vanbaalenii* strain PYR-1) (Khan *et al.* 2002) was 97.8%. The majority of the sequence differences were observed within the first 200 bp. (data not shown).

2.4.2. Growth kinetics and carbon utilization pattern

The growth kinetics and subsequent culturing of the strain were carried out using YTS1000 broth and plates. Determination of the optimum growth temperature of S65 was performed at temperatures of 10, 20, 25, 30, and 35°C in YTS1000 broth, pH 7.0 (Figure 2.1a and 2.1b). Optimal growth was observed at 30°C. This strain did not grow at 35°C, suggesting that it cannot survive in the human body, and is unlikely a human pathogen. Growth at 10°C was also examined since the strain was isolated from a site in northern Quebec. No significant growth was observed in the first 150 hr at 10°C, whereas when incubated at 20 to 30 °C the stationary phase was achieved within 150 hr. After 600 hr of incubation at 10°C, maximum growth was achieved.

Functional diversity of S65 was determined using Biolog GP2 Microplates. After 24 hr of incubation, the OD_{590} was measured for each of the substrates. Substrates with an OD_{590} greater than the control (water) by a factor of 1.4 were considered as positive for S65. S65 grew well on Tween-80, which is a typical substrate for mycobacteria. Glycerol, which is commonly added to selective mycobacterial media, was not utilized by this strain (Appendix 1-3).

2.4.3. Mineralization of PAHs

To screen for the ability of S65 to degrade PAHs, mineralization of PAHs with varying numbers of rings (2 to 4 rings) and structures (linear, angular, and cluster) was monitored over a two-week period. All microcosms were prepared with MSM broth supplemented with PAHs as sole carbon sources. The PAHs tested were: anthracene (linear and three-ring), naphthalene (linear and two-ring), fluorene (linear and heterocyclic), phenanthrene (angular and three-ring), and pyrene (cluster and four-ring). The results are shown in Figure 2.2. Pyrene mineralization begun immediately after the inoculation of the bacterium; a mineralization extent of 45% was reached after the first 24 hr, which eventually increased to 60% within 96 hr. No further mineralization of pyrene was observed after this point. No lag phase was observed prior to pyrene mineralization even though the inoculum was not prepared under induced conditions. On the other hand, there was a significant lag phase prior to the onset of mineralization of phenanthrene. In

the case of phenanthrene, no significant mineralization was observed for the first 72 hr. Mineralization was observed during the next 72 hr and finally reached a maximum extent of 60%. Low-molecular-weight (LMW) PAHs (anthracene, naphthalene, and fluorene) were not mineralized by this strain. An additional mineralization assay using radiolabelled fluoranthene was conducted. Fluoranthene was mineralized without undergoing a lag phase but a maximum extent of only 20% was attained (Figure 2.3).

The influence of other PAHs on the mineralization of pyrene by S65 was determined. Non-radiolabeled PAHs (phenanthrene, fluoranthene, benzo[a]pyrene, and benz[a]anthracene, individually, or as a mixture) were added into the microcosms containing radiolabeled pyrene. This assay was carried out under both induced and non-induced conditions. Initiation of pyrene mineralization was observed immediately after S65 was introduced into the microcosms containing benzo[a]pyrene and benz[a]anthracene in spite of pre-growing S65 in the absence of pyrene (non-induced) (Figure 2.4A). Mineralization of pyrene in the presence of benzo[a]pyrene, and benz[a]anthracene, was more than 30 % in the first 15 hr. Pyrene mineralization with benzo[a]pyrene reached a maximum extent of 57% after 90 hr. Pyrene mineralization in the presence of phenanthrene showed a long lag phase in the first 30 hr but reached 54% mineralization. A lag phase was also observed in pyrene mineralization when fluoranthene and a mixture of these PAH substrates were present. In the presence of fluoranthene and the PAH mix as co-substrates, the extents of pyrene mineralization were below the reference (^{14}C-pyrene only).

Differences in pyrene mineralization were observed in microcosms containing phenanthrene and fluoranthene under the induced condition (Figure 2.4B); a lag phase was not observed in the presence of phenanthrene, fluoranthene and the PAH mix during pyrene mineralization with induced cultures. A 25% increase in the mineralization of pyrene in the first 15 hours was observed in microcosms containing benz[a]anthracene. An increase in mineralization extent was also seen in the presence of other PAHs. Microcosms containing benzo[a]pyrene and a mixture of the PAHs also showed a 20% increase in the mineralization extent. An increase in the extent of mineralization in microcosms with the mixture of the PAHs was significant compared to the extent in non-induced cultures. The mineralization extent surpassed the reference and reached a

maximum of 67% which was equivalent to the highest mineralization extent (68%) produced in microcosms with benzo[a]pyrene. The overall extent of pyrene mineralization was also greatly enhanced in other microcosms with induced cultures. The extent of mineralization eventually reached a maximum of 68% in microcosms containing benzo[a]pyrene, phenanthrene, benz[a]anthracene, and the PAH mixture. Pyrene mineralization was suppressed in the presence of fluoranthene, to a lower extent than the reference, however, no lag phase was produced with induced cultures. The rate of pyrene mineralization was much faster with induced cultures. The final mineralization extent was 46%, which was not significantly different from the extent (42%) with non-induced cultures.

2.4.4. Genomic DNA extraction

The standard protocol for extraction of bacterial genomic DNA by Ausubel *et al.* (1998) was modified due to the difficulties encountered in obtaining genomic DNA from this strain. Without the addition of a bead-beating step to disrupt cell walls, the cells remained intact and no DNA was released (living cells were observed under the microscope after 20% SDS treatment). Two large plasmids were also detected in S65 (data not shown). An aliquot of the plasmid DNA was loaded beside digested genomic DNA for Southern hybridization analysis, but no signal obtained from the plasmid DNA using the *nidA* gene probe. The role of these plasmids in S65 has not been determined.

2.4.5. PCR analysis for the presence of catabolic genes in S65.

To understand the genetic diversity of PAH degradation, the pyrene catabolic genes of S65 were compared by PCR to three known bacterial ring-hydroxylating dioxygenase genes which degrade naphthalene (*ndoB*) (Kurkela *et al.* 1988), phenanthrene (*phnAc*) (Laurie and Lloyd-Jones 1999), and pyrene (*nidA*) (Khan *et al.* 2001). Primers specific to each gene were designed and used at various annealing temperatures (50 – 70°C). No amplification of the *ndoB* gene of *Pseudomonas putida* or the *phn*Ac gene of *Burkholderia* sp. strain RP007 was detected in S65 under the above

annealing conditions. With the *nid*A gene, a strong and intense band was amplified at an annealing temperature of 65°C (data not shown).

2.4.6. Screening for pyrene catabolic genes and nucleotide sequencing

The genomic DNA of S65 was digested with nine different restriction enzymes and screened for the *nidA* gene. Southern hybridization of S65 with a probe made with a 500 bp PCR product of the *nidA* gene amplified using S65 DNA as a template revealed the presence of a putative *nidA* gene in several sites of the S65 genome (Figure 2.5). Interestingly, multiple bands appeared in the genomic DNA digested with individual restriction enzymes (*Bam*HI, *Eco*RI, *Eco*RV, *Sac*I, and *Sac*II). Fragments of 7 kb and 14 kb digested with *Bam*HI hybridized strongly with the *nidA* probe. Four hybridization signals were observed in the *Eco*RI digest, but the signal having the largest DNA fragment could be the result of incomplete digestion. Genomic DNA digested with *Eco*RV gave two positive hybridization signals at 7 kb and 11 kb. *Sac*I digested genomic DNA produced three signals and *Sal*I had one signal. A restriction digestion site map of the 500 bp of the *nidA* gene revealed that *Sac*I and *Sal*I were able to cut once within the fragment. Fragments of genomic DNA produced by *Sac*II digestion gave two low molecular weight signals of 1 kb and 1.6 kb. A fragment of approximately 700 bp produced a signal in genomic DNA digested with *Spe*I. Although S65 was found to contain two large plasmids (data not shown) a positive reaction was not observed with the 500 bp *nidA* gene probe. The 7 and 11 kb fragments generated by *Eco*RV were used for further characterization of the pyrene degradation genes in S65.

Twenty clones with a positive *nidA* signal were obtained from the 7 and 11 kb fragments of genomic DNA clones int *Eco*RV sites and screened for insert DNA. Eighteen clones with DNA inserts were further checked by PCR amplification with the *nidA* gene primers. These clones were also digested with *Stu*I and *Sac*I, separately. Two 7 kb clones and one 11 kb clone with good PCR amplification and distinctive RFLP digestion patterns with *Stu*I or *Sac*I were selected. Further RFLP analysis of the two 7 kb clones with *Sac*I and *Ban*II (double digests) showed they had the same DNA inserts in the opposite orientation (data not shown). Therefore, two clones, each from one 7 kb and the other 11 kb were selected and sequenced. Complete sequences for both clones are shown

in Appendix 1-4 and 1-5, respectivvely, and the gene organization of both clones are shown in Figure 2.6. GenBank accession numbers are AF546904 and AF546905, respectively.

ii) Bacterial ring hydroxylating dioxygenase genes.

OrfN1, *orfN5*, *orfP3*, and *orfP7*, were predicted to encode for the α subunits of bacterial ring-hydroxylating dioxygenase genes because the consensus sequence Cys-X_1-His-X_{16}-Cys-X_2-His of a Rieske-type [2Fe-2S] cluster binding site for a bacterial ring-hydroxylating dioxygenase was identified in the deduced amino acid (a.a.) sequences of these *orf*s. The nucleotide sequence of *orfN1* (1,368 bp) showed 99.5% identity to the *nidA* gene of *Mycobacterium* sp. strain PYR-1. Comparison of nucleotide sequences of *orfP3* (1,380 bp) and the *nidA* gene of PYR-1 revealed that the identity of *orfP3* to the *nidA* gene was less, at 89%, with clear differences, especially at the 5' end. *OrfP3* was designated as the *pyrene dioxygenase A* (*pdoA*) gene as it is suspected to function as an initial dioxygenase gene in pyrene degradation. PCR was carried out with primers designed specific only for the *pdoA* and *nidA* genes using PYR-1 and S65 genomic DNA as templates. The result showed that there was no amplification of the *pdoA* gene with PYR-1 but there was with S65 genomic DNA (data not shown). Third and fourth α subunit dioxygenase genes were present in *orfN5* (*nidX*) and *orfP7* (*pdoX*). The nucleotide sequence of these α subunit dioxygenase genes did not match with any of the known genes in the databases, but their deduced amino acid (a.a.) sequences showed 60% homology to a hypothetical α subunit dioxygenase in a dioxin-degrading bacterium, *Sphingomonas* sp. strain RW1 (Armengaud *et al.* 1998). A β subunit dioxygenase gene, *orfP2*, which had 89% similarity to the *nidB* gene of PYR-1. This gene was designated as *pdoB* since it was found in the same locus as *pdoA*.

ii) Alcohol dehydrogenase genes

Two alcohol dehydrogenase sequences were predicted just downstream of the α subunit dioxygenase genes in both clones. A gene for *orfN2* (*nidC*) and *orfP4* (*pdoC*) encoded for a short-chain alcohol dehydrogenase and *orfN3* (*nidH*) and *orfP5* (*pdoH*) for a zinc-containing alcohol dehydrogenase. No significant alignment with any known

genes was detected in any of these *orf*s; at the a.a. level, they showed a relationship to known alcohol dehydrogenases, (OrfN2 and OrfP4 to short-chain alcohol dehydrogenases and OrfN3 and OrfP5 to zinc-containing alcohol dehydrogenases, respectively) but the similarity was only 40%. These findings suggested that these genes are new members of the short-chain and the zinc-containing alcohol dehdrogenases.

iii) Other genes found with the pyrene catabolic gene cluster

Two open reading frames (*orfN4* and *orfP6*) were found, flanked by the zinc-containing alcohol dehydrogenase gene and the hypothetical large subunit dioxygenase gene. They showed no similarity to known genes in the GenBank database. The deduced polypeptide of *orfP1* exhibited the DDE motif, highly conserved in the IS3 family (Poland and Chandler 1995) and showed a close relationship to tranposases in the IS3 family. It had a high homology (68%) to a putative transposase from a deep marine bacterium, *Micrococcus* sp. strain 28. Its functional activity, however, is questionable. A stop codon was found in a middle of the sequence, which would cause the polypeptide to be produced as two fragments.

iv) A putative promoter

A putative promoter region (TCTCTCTTGACGCGCAAGTGAGATCTGGC TAACATTCTCTACAGCGGAGGACTTATCTG) was determined using promoter prediction software available at the Berkeley Drosophila Genome Project (BDGP) web site (http://www.fruitfly.org/seq-tools/promoter.html), 57 bp upstream of the *pdoB* gene. The transcription of genes was predicted to start at the nucleotide underlined in the region. A ribosomal binding site (SD), GGAG, was also found at –10 of the putative initiation codon of the *pdoB* gene.

2.5. Discussion

Mycobacterium sp. strain S65 was isolated from a site contaminated with jet-fuel in Sept-Iles, northern Quebec. Based on 16S rDNA sequence analysis, the closest known relatives of S65 are *Mycobacterium* sp. strains T103 and T104 (99% similarity), which were isolated from rock surface biomass in a freshwater stream contaminated with

toluene (Tay *et al.* 1998). However, S65 can be distinguished from these strains by its ability to degrade HMW-PAHs. Strain S65 is also closely related to *Mycobacterium* sp. strains RJGII-135 (98% similarity) and PYR-1 (97.8% similarity) (Govindaswami *et al.* 1995). This result is significant because strains RJGII-135 and PYR-1 are both HMW-PAH degrading bacteria. S65 also falls within the fast-growing *Mycobacterium* clade based on its identical sequence to that of RJGII-135 and PYR-1 between positions 451 and 482 (*E. coli* numbering) of the 16S rDNA (Govindaswami *et al.* 1995). The fast-growing mycobacteria are considered as non-pathogenic to humans and other animals (Hartmans and de Bont 1992) suggesting that since S65 did not grow at 35°C it would not survive in the human body. Although S65 grows slowly on YTS1000 plates (it takes 2 weeks to see visible colonies on the plates) it has not been tested for growth on mycobacteria standard media in which the growth kinetics of most mycobacteria have been tested. S65, however, can be distinguished from RJGII-135 and PYR-1 at the physico-chemical level. S65 is able to grow at temperatures ranging from 10°C to 30°C but not at 35°C, whereas strains RJGII-135 and PYR-1 are able to grow at 37°C. Also, S65 does not grow in rich media such as TSB (trypticase soy broth). Most importantly, the mineralization kinetics of S65 are distinctive to those of RJGII-135 and PYR-1 (discussed below). These differences suggest that S65 is unique and may be a new member of the xenobiotic-degrading mycobacteria.

Mycobacterium sp. strain S65 was able to rapidly degrade phenanthrene and pyrene (60% mineralization), as sole carbon and energy sources. S65 was also able to degrade fluoranthene to a limited extent (20% mineralization). The well-known pyrene degrading bacterium, *Mycobacterium vanbaalenii* strain PYR-1 requires a low concentration of nutrient supplements in addition to a mineral basal medium in order to degrade pyrene and other PAHs (Heitkamp and Cerniglia 1988). S65, however, is not able to degrade low-molecular-weight (LMW) PAHs including anthracene, naphthalene, and fluorene (Figure 2.2.). We observed from the mineralization results that S65 has a preference for degrading cluster (pyrene) and angular PAH (phenanthrene and fluoranthene) molecules rather than linear (naphthalene, anthracene, and fluorene) PAH molecules. This substrate specificity of S65 is very similar to that of *Mycobacterium flavenscens* (Dean-Ross and Cerniglia 1996) and *Mycobacterium* sp. strain AP1 (Vila *et*

al. 2001). Strain S65, however, degrades those three PAHs to a greater extent than *M. flavenscens*. No comparison can be made to the strain AP1 because the extent of mineralization for these substrates has not been reported. One hypothesis is that S65 preferentially attacks a bay-region and a K-region of PAH molecules as an initial ring-cleavage site. Various proposed pyrene degradation pathways in a number of *Mycobacterium* strains supports this hypothesis (Dean-Ross and Cerniglia 1996; Heitkamp *et al.* 1988; Vila *et al.* 2001). All the pathways agree that an initial hydroxylation of pyrene occurs at the C-4, C-5 positions, located in the K-region. One of the cleavage products of pyrene, 4-phenanthroic acid, has the structure of phenanthrene with a –COOH at the C-4 position. A subsequent hydroxylation occurs at the C-3, C-4 positions, adjacent to the bay-region, of 4-phenanthroic acid. The incorporation of dioxygen at these positions has also been seen for phenanthrene degradation in bacteria (Iwabuchi and Harayama 1997).

A 3-day lag phase was observed in phenanthrene degradation by S65 (Figure 2.2) despite the fact that phenanthrene was mineralized to the same extent as was pyrene. It is possibile that the recognition of the molecular structure of pyrene is required to orchestrate the sequence of enzymatic degradation in the cell (Cenci *et al.* 1999). Phenanthrene can be broken down by S65 not because it is a primary carbon source, but because it has a structure similar to 4-phenanthroic acid, one of the intermediate metabolites of pyrene degradation. It is possible that NidA (the large subunit of the naphthalene inducible dioxygenase) is a transmembrane protein since an overexpressed NidA was found in the cell pellets rather than in the supernatants (Khan *et al.* 2001). If this is so, then the molecular structure of pyrene plays an important role in the activation of NidA and for entry into the cells since the solubility of pyrene is very low. Phenanthrene, on the other hand, has relatively high solubility, making its entry into the cells easier. NidA does not recognize phenanthrene, therefore it is not activated by the entrance of phenanthrene. No induction of an enzymatic cascade occurs, and a lag phase is observed. This active transport of PAHs and the involvement of membrane-bound enzymes for cellular uptake of PAHs have been seen in *Pseudomonas fluorescens* LP6a (Bugg *et al.* 2000).

Mineralization of fluoranthene does not have a lag phase (Figure 2.3). Fluoranthene may be initially metabolized by the same dioxygenase as for pyrene catabolism or another dioxygenase (discussed below) but the enzymes required for further metabolism of fluoranthene may not be present in S65. It is also that intermediate metabolites of fluoranthene could be toxic to the cells hence the cells may not survive. This might explain why fluoranthene mineralization only reached 20%. Another possibility is that because only the third position of carbon is radiolabelled in fluoranthene it may not accurately measure the total mineralization extent by tracing $^{14}CO_2$ evolution.

The biodegredation kinetics of pyrene by S65 were investigated in binary and multiple substrate systems. Mineralization on pyrene alone was used as a reference and pyrene was the only radiolabelled carbon source. The purpose of this experiment was to examine the interactions of pyrene with other PAH substrates since PAHs are always present in nature as a mixture (Guha *et al.* 1999). From previous studies on the behaviour of multisubstrate PAH degradation, the biodegradation of the more degradable and abundant compounds is reduced due to the presence of other PAHs (competitive inhibitors), but the biodegradation of highly recalcitrant PAHs is increased due to the simultaneous biomass growth on multiple substrates (enhancers). This study was conducted under non-induced and induced conditions to examine the behaviour of pyrene catabolic enzymes in the presence of other PAH substrates. A lag phase was observed when phenanthrene was added as a co-substrate with the non-induced cultures (Figure 2.4A), as was observed during phenanthrene mineralization. Phenanthrene may act as a competitive enhancer because pyrene was mineralized to a higher extent than the reference, which was pyrene alone. With fluoranthene and a PAH mixture, pyrene mineralization did not reach the same extent as the reference. Fluoranthene, which was 20% mineralized by S65 as a sole carbon and energy source without a lag phase (Figure 2.3), may act as a competitive inhibitor, or the metabolites may be toxic to the cells so that the cells died off before it reached its maximum pyrene mineralization. The reduction of pyrene mineralization could be explained by the above reasons despite the presence of other enhancers in the PAH mixture. Under induced conditions, the PAH mixture did not affect pyrene mineralization. Moreover, the final mineralization extent

was as high as that observed in pyrene mineralization in the presence of benz[a]anthracene, benzo[a]pyrene, or phenanthrene. The extent of pyrene mineralization was unchanged in the presence of fluoranthene. It is believed that synergistic effects of other PAH enhancers overcame the inhibition under induced conditions. These results support the study done by Bouchez *et al.* (1993), which suggests that cometabolism of other PAHs is commonly inhibited when the added PAHs are more water-soluble than the test PAH (Bouchez *et al.* 1993). These phenomena were also observed during this study. When less water-soluble PAHs, i.e., benz[a]anthracene or benzo[a]pyrene, were added as cosubstrates, pyrene was mineralized to a greater extent than when more water-soluble PAHs, i.e., fluoranthene were added.

Analysis for the presence of plasmids showed that there are two large plasmids in S65 (data not shown). In Southern hybridization, the large plasmids did not show hybridization with the *nidA* gene PCR probe, indicating that it is likely that pyrene catabolic genes are located in the chromosome of S65. The function of the large plasmids is yet to be determined.

Multiple signals from Southern hybridization of S65 genomic DNA, digested with individual restriction enzymes, with the *nidA* gene of PYR-1 indicated that at least two loci encoding for the *nidA* or *nidA* homologuesare located in the chromosome. Identification of the *nid* and *pdo* loci in the *Eco*RV digested S65 genome demonstrates that a pyrene catabolic pathway is encoded in multiple sites. As seen in most catabolic pathways in bacteria, the genes for pyrene degradation pathway are also clustered (Figure 2.5). Interestingly, the organizations of the pyrene degrading genes are identical in both loci, indicating that the two loci may be related to each other. The presence of a transposase gene upstream of the pyrene catabolic genes in the *pdo* locus suggests that the *pdo* locus might have originated from the *nid* locus. The horizontal transfer of catabolic genes (or functions) among bacteria or within the same bacterium, facilitated by transposable elements, is common in nature (Harayama and Kok 1992). This hypothesis is further supported by the rate of mutations of the catabolic genes in the *pdo* locus. The difference between the genes in the *pdo* locus and their corresponding genes in the *nid* locus is consistent at both the nucleotide and amino acid levels. At the nucleotide level, the difference is around 12% whereas at the amino acid level, it decreases to 3%. The

gene arrangement of the pyrene degrading genes in S65 is unique to that previously reported (Goyal and Zylstra 1996; Kiyohara *et al.* 1994; Laurie and Lloyd-Jones 1999; Saito *et al.* 1999). What is most intriguing in the arrangement is that two alcohol dehydrogenase genes are located adjacent to each other. This is the first study that has shown that two alcohol dehydrogenase genes are encoded together.

The other putative α subunit dioxygenase gene, *nidX* or *pdoX*, has no similarity at the nucleotide level to any known genes in the GenBank database, but the deduced amino acid sequences were 60% identical to a hypothetical α subunit dioxygenase (OrfG1) in *Sphingomonas* sp. strain RW1 (Armengaud *et al.* 1998). RW1 is known as a dioxin degrading bacterium and its dioxin dioxygenase gene, *dxnA1*, is capable of attacking the aromatic ring of dibenzofuran at a bridge position, resulting in the production of the metabolite, 2,2',3-trihydroxybiphenyl (THB). This metabolite has a similar structure to the novel pyrene metabolite, 6,6'-dihydroxy-2,2'-biphenyl dicarboxylic acid, recently described in *Mycobacterium* sp. strain AP1 by Vila *et al.* (2001). Knowing that NidX and PdoX have high a.a. sequence similarity to ORFG1, which is categorized in the same class as DXNA1, NidX and PdoX may have a role in the formation of 6,6'-dihydroxy-2,2'-biphenyl dicarboxylic acid in pyrene catabolism.

The localization of two alcohol dehydrogenase genes adjacent to each other and clustered within the pyrene catabolic genes created some confusion as to which gene is involved in the dehydrogenation of *cis*-4,5-dihydroxy-4,5-dihydropyrene. Because most dehydrogenase genes involved in the initial dehydrogenation of aromatic rings belong to the short-chain alcohol dehydrogenase family (PROSITE accession number PDOC00060), the *nidC* and *pdoC* genes are more likely to play a role as the *cis*-4,5-dihydroxy-4,5-dihydropyrene dehydrogenase gene. Another dehydrogenase gene belonging to the classical zinc-containing alcohol dehydrogenase family encoded by the *nidH* and *pdoH* genes, has no known function. The dehydrogenase genes are separated by only 57 bp from *nidC* and *pdoC*, respectively, therefore are possibly co-transcribed. It is speculated that there is another alcohol metabolite in the S65 pyrene catabolic pathway and the *nidH/pdoH* genes may be involved in the dehydrogenation of the yet-to-be discovered alcohol metabolite. In fact, the *xylB* gene, involved in dehydrogenation of benzyl alcohol to benzaldehyde in *Pseudomonas putida* belongs to the zinc-containing

alcohol dehydrogenase family (Reid and Fewson 1994). Moreover, Rehmann *et al.* (1998) identified 2-carboxy-benzaldehyde as one of the metabolites in pyrene degradation by *Mycobacterium* sp. strain KR2. It is worth mentioning that alcohol attached to aromatic rings is dehydrogenated by enzymes in the short-chain alcohol dehydrogenase family, and alcohol attached to a substituent on aromatic rings is dehydrogenated by enzymes in the long-chain alcohol dehydrogenase family.

In *Mycobacterium vanbaalenii* strain PYR-1, an aldehyde dehydrogenase gene, *nidD*, was found upstream of the *nidB* gene. In the *pdo* locus, a putative promoter site was found, but no *nidD* homologue was located upstream of the *pdoB* gene. It is uncertain whether there is a promoter between the *nidD* and *nidB* genes in PYR-1 since the presence of a promoter was not mentioned (Khan *et al.* 2001). There is a 1,283 bp region between the transposase and the putative promoter in the *pdo* locus but it does not appear to encode for any genes. A transposase gene was found upstream of the pyrene catabolic genes in the *pdo* locus. Based on its sequence, it belongs to the IS3 family of bacterial transposases. However, a stop codon was found in the middle of the transposase gene, which may indicate that the transposase has lost its function. It is suggested that the transposase was once active and the pyrene catabolic genes were translocated from the *nid* locus to the *pdo* locus.

In this study, the pyrene degradation potential of *Mycobacterium* sp. strain S65 was characterized and the genetic elements of the upper pathway for pyrene catabolism were partially identified. A novel locus, *pdo*, encoding an upper pathway for pyrene degradation was also found in S65 along with the *nid* locus as found in PYR-1. The pyrene catabolic genes encoded in the *pdo* locus are homologous to those in the *nid* locus, but are distinct enough that no PCR amplification was obtained with PYR-1 genomic DNA using *pdoA* primers. This finding of new pyrene catabolic genes expands the current understanding of the genetic mechanism of bacterial HMW-PAH degradation.

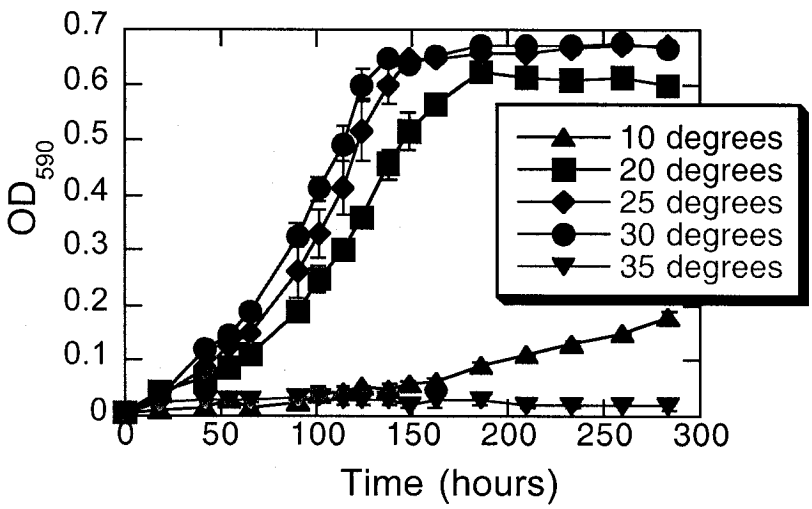


Figure 2.1A. Growth of S65 in YTS1000, pH 7.0 at different temperatures. The rate of growth was expressed as the measurement of optical density at 590 nm. Data points are the average of triplicate samples and error bars represent one standard deviation.

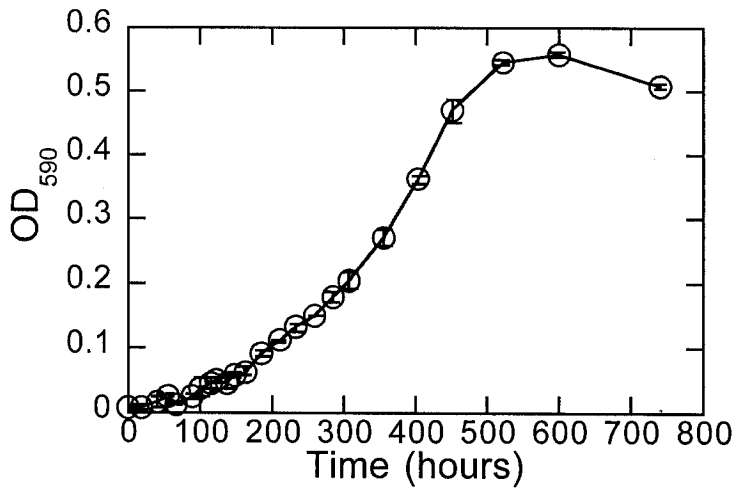


Figure 2.1B. Growth of S65 at 10°C in YTS1000, pH 7.0.

S65 was inoculated into YTS1000 in triplicate and incubated at 10°C until the growth reached stationary phase. Data points are the average of triplicate samples and error bars represent one standard deviation.

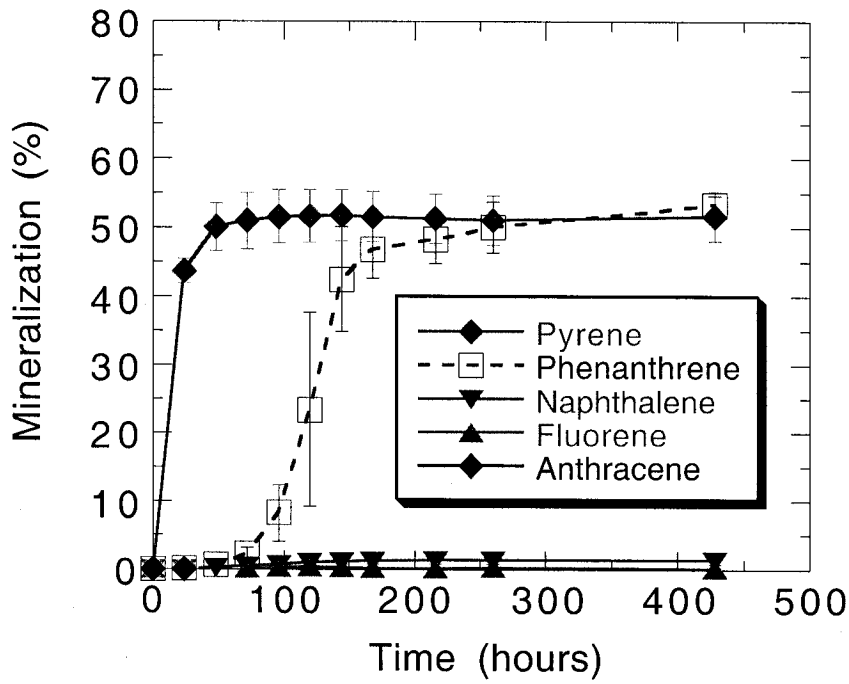


Figure 2.2. Time course for mineralization of various PAHs by *Mycobacterium* sp. strain S65. Strain S65 was pre-grown in a low nutrient broth to the mid-log phase and the culture was washed with H_2O, to remove residual nutrients, prior to inoculation. Data points are the average of triplicate samples and error bars represent one standard deviation.

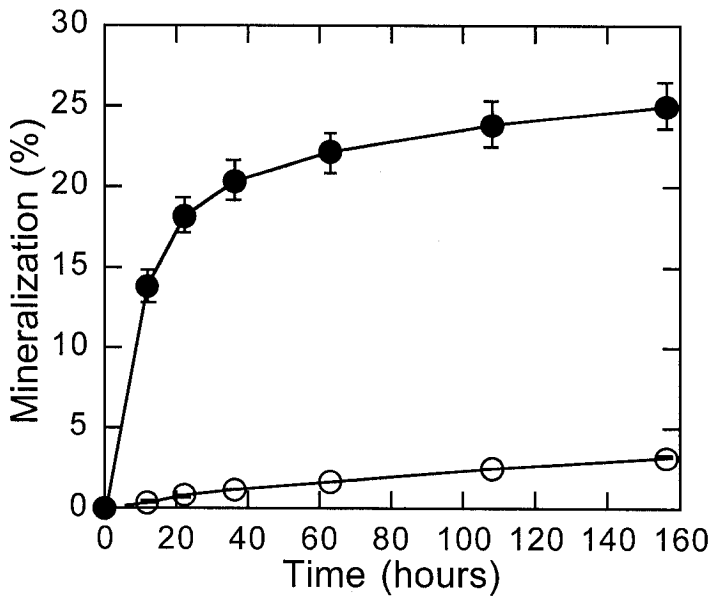


Figure 2.3. Time course of fluoranthene mineralization by *Mycobaceterium* sp. strain S65. Fluoranthene mineralization is shown in a viable culture (●) and a heat-killed control (○). Data points are the average of triplicate samples and error bars represent one standard deviation.

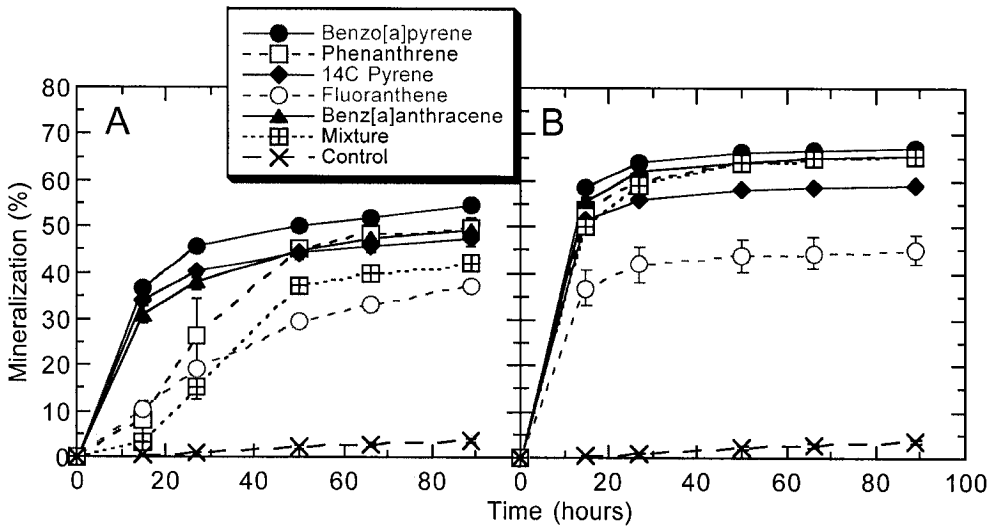


Figure 2.4A and B. Time course of pyrene mineralization in the presence of various PAHs by *Mycobacterium* sp. strain S65 under the non-induced (A) and induced (B) conditions. Mineralization of pyrene in the presence of cold phenanthrene, fluoranthene, benz[a]anthracene, or benzo[a]pyrene, added to a concentration of 1 mg/L in each microcosm, was measured. The pyrene mineralization without any cold substrate was used as a control. Data points are the average of triplicate samples and error bars represent one standard deviation.

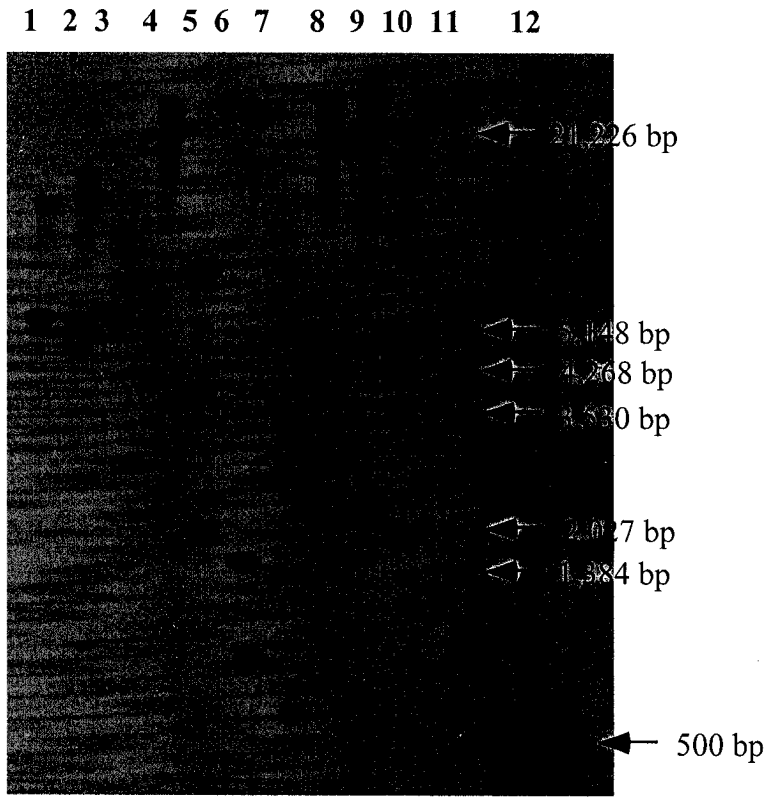


Figure 2.5. Southern hybridization of S65 genomic DNA using the pyrene catabolic gene, *nidA*. Genomic DNA of S65 was digested with various restriction enzymes and was tested for the presence of the *nidA* gene from *Mycobacterium* sp. strain PYR-1. S65 genomic DNA was digested with: lane1, *Bam*HI; lane 2, *Eco*RI; lane 3, *Eco*RV; lane 4, *Hind*III; lane 5, *Sac*I; lane 6, *Sac*II; lane 7, *Sal*I; lane 8, *Spe*I; lane 9, *Xba*I; lane 10, DIG Marker; lane 11, S65 plasmid; lane 12, *nidA* PCR probe.

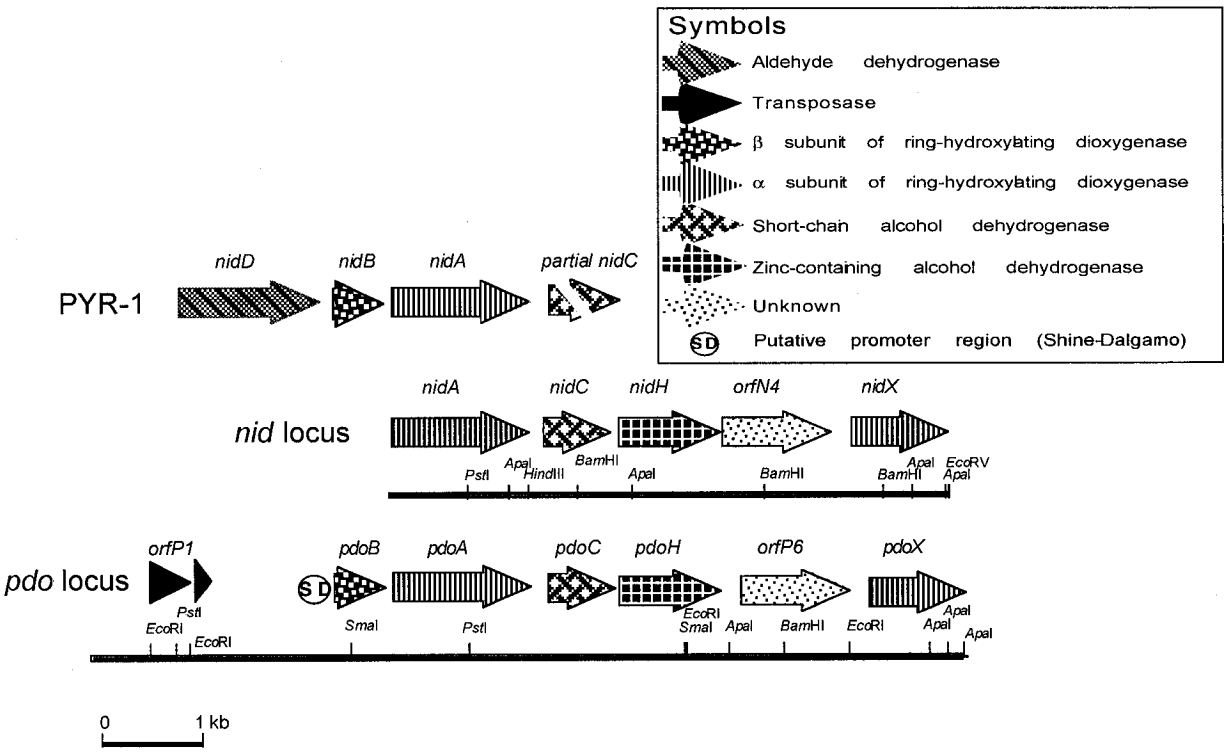


Figure 2. 6. Genetic organizations of loci encompassing the genes identified in this study. The positions and orientations of the orfs are shown by full arrows.

Table 2.1. Genes and gene products of *nid* locus and *pdo* locus

ORF	Gene	length (nt)	Putative gene function	identity (%) nucleotide	protein	Reference
N1	*nidA*	1,368	α subunit pyrene dioxygenase	99.5	100	*nidA, Mycobacterium vanbaalenii* strain PYR-1, AF249301
N2	*nidC*	681	*cis*-4,5-dihydroxy-4,5-dihydropyrene dehydrogenase	0		*Deinococcus radiodurans* AE001991
N3	*nidH*	1,026	Dehydrogenase	0	40	*Pseudomonas putida* L35343
N4		941	Unknown	0	16	Hypothetical protein *Ralstonia solanacearum* AL646084
N5	*nidX*	1,187	α subunit dioxygenase	0	60	*Sphingomonas* sp. strain RW1 AJ223219
P1		987	transposase	0	60	Putative transposase *Micrococcus* sp. 28 AY034092
P2	*pdoB*	510	β subunit dioxygenase	89	91	*Mycobacterium vanbaalenii* strain PYR-1, AF249302
P3	*pdoA*	1,376	α subunit pyrene dioxygenase	89	90	*Mycobacterium vanbaalenii* strain PYR-1 *Mycobacterium* sp. strain S65, (this study)
P4	*pdoC*	681	*cis*-4,5,-dihydroxy-4,5-dihydropyrene	89	98	*Mycobacterium* sp. strain S65, (this study)
P5	*pdoH*	1,026	Dehydrogenase	0	40	*Pseudomonas putida* L35343

				88	98	*Mycobacterium* sp. strain S65, (this study)
P6		942	Unknown	87		*Mycobacterium* sp. strain S65 (this study)
P7	*pdoX*	1,180	α subunit pyrene dioxygenase	0	60	*Sphingomonas* sp. strain RW1, AJ223219
				93	98	*Mycobacterium* sp. strain S65, (this study)

Connecting Text

Based on the mineralization results obtained in Chapter 2, *Mycobacterium* sp. strain S65 was studied as a potential pyrene and phenanthrene remediation agent in a PAH contaminated soil. This study included an optimization of bioaugmentation conditions, determination of the efficiency of bioremediation of phenanthrene and pyrene from the soil using chemical analysis, and the survival of S65 in the soil using DGGE analysis. Michiei Sho performed all of the experimental work, accompanying interpretation of data, and writing of the manuscript. Drs. Charles W. Greer and Chantal Hamel provided valuable suggestions to improve this study.

Chapter 3. *Mycobacterium* sp. Strain S65 as a Remediator of PAH Degradation in Soils

The work presented in this chapter is being compiled into a manuscript for potential publication. The title of the manuscript is "*Mycobacterium* sp. Strain S65 as a Remediator of PAH Degradation in Soils." I was the author and performed the experimantal work. The co-authors participated in the design of the experiments, in analysis of the data and revision to the manuscript are Drs. Charles W. Greer and Chantal Hamel.

3.1. Abstract

The pyrene degrading bacterium, *Mycobacterium* sp. strain S65 was introduced, as a bioaugmentation agent, into a soil historically contaminated with polycyclic aromatic hydrocarbons (PAHs). Previously, S65 indicated its ability to degrade high molecular weight (HMW) PAHs such as fluoranthene and pyrene, as well as a low molecular weight (LMW) PAH, phenanthrene, in a microcosm setting (Chapter 2). The bioaugmentation study was continued for 90 days, and six different treatments were tested to determine optimum degradation kinetics for S65 in the soil. The survival of S65 in the soil was determined using a combination denaturing gradient gel electrophoresis (DGGE)-hybridization technique. Soil DNA extracts along with S65 genomic DNA were subjected to PCR using universal bacterial 16S rDNA primers and the PCR amplicons were separated on the basis of sequence differences by DGGE. The denaturing gradient gel was blotted on a membrane and hybridization was carried out at 65°C using the S65 16S rDNA PCR amplicon as a probe. Multiple signals were detected in the soil samples, indicating that the 16S rDNA probe was not specific enough to detect only S65. The remediation of the PAHs, phenanthrene and pyrene, from the soil was routinely examined by chemical analysis (Soxhlet extraction) of the soil samples over time. The chemical analysis showed that no significant decrease in the concentration of these PAHs was observed in the soil during bioaugmentation. These results indicate that S65 was not successful as a bioremediation agent under the conditions that were used in the present study.

3.2. Introduction

Remediation of sites contaminated with xenobiotic compounds such as polycyclic aromatic hydrocarbons (PAHs), mainly produced during the combustion of organic materials like fossil fuels, has become important due to the increasing awareness of the potential adverse effects of these pollutants on human health and the environment.

Bioremediation is a method to clean up the contaminated sites using the potential of microorganisms to degrade organic pollutants (Head 1998). This method has received considerable attention as an alternative method over the conventional remediation techniques such as chemical and physical processes because of the cost effectiveness and metabolic repertoire of natural microorganisms for various organic compounds.

A considerable amount of research has been carried out to optimize bioremediation conditions. Many PAHs are degradable by various microorganisms (Heitkamp and Cerniglia 1988; Hughes *et al.* 1997; Juhasz *et al.* 1996) but when they are introduced at contaminated sites, the rates are much lower than the rates expected from the results obtained in a laboratory setting. This is mainly due to the limited accessibility of the pollutants to microorganisms in an environmental setting like soil. PAHs are hydrophobic compounds, and tend to be adsorbed on soil matrices by hydrogen bonding. Solubility of the PAHs in water is minimal with a decrease in solubility as a number of rings increases. This hydrophobic characteristic is a problem in a bioremediation process because microorganisms are not capable of taking up PAHs unless these compounds are dissolved in aqueous form (Harms and Bosma 1997) Therefore, high molecular weight (HMW) PAHs are more recalcitrant in the environment and toxicity increases as the number of rings increases (Pickering 2000). Application of surfactants in contaminated soils is one of the methods to overcome the problem. Surfactants increase the bioavailability of PAHs to microorganisms by increasing their aqueous solubility (Thibault *et al.* 1996; Tiehm 1994; Volkering *et al.* 1995; Willumsen and Karlson 1998).

Humidity and nutrient availability also greatly influence microbial activity on PAH degradation. Cattaneo *et al.* (1997) reported that the biodegradation activity of an introduced bacterium, *Burkholderia cepacia* strain BRI6001L on 2,4-dichlorophenoxyacetic acid decreased when the moisture level decreased below 15% of the water holding capacity of the soil, and no degradation was observed below 6% in soil

columns. Nutrients, especially nitrogen and phosphorus are also important to microorganisms for activity and growth (Atlas 1977; Cerniglia 1992; Wilson and Jones 1993). An arctic soil contaminated with petroleum hydrocarbons was subjected to a bioremediation assessment and nutrients (nitrogen and phosphorus) were added in the form of a commercial fertilizer (Whyte *et al.* 1999). The concentration of total petroleum hydrocarbons in the soil decreased to a greater extent when the fertilizer was added during the assessment.

When a microorganism is introduced into a contaminated soil to enhance the biodegradation rate (bioaugmentation), the survival and in situ activity of the inoculated organism is a key to the success of the bioremediation. It has been shown that the population size of the introduced bacterium declines rapidly following introduction due to the scarcity of available nutrient sources and the hostility of the soil environment (van Veen *et al.* 1997). Many detection methods have been developed to monitor survival and activity of introduced microorganisms. The most common detection method is to genetically tag the organisms by incorporating marker genes such as a *lac-lux* reporter system, green fluorescent protein (*gfp*), or antibiotic resistant genes (Boon *et al.* 2000; Errampalli *et al.* 1999; Flemming *et al.* 1994; Masson *et al.* 1993). However, these marker genes are not always stable and horizontal transfer of these genes in the environment needs to be considered. Also, it takes a long time to incorporate such a marker systems into a bacterial host. Another consideration is that the act of marking the organism may alter the expression of other genes and make the organism less competitive in the natural environment.

Denaturing gradient gel electrophoresis (DGGE) has been used frequently to determine microbial diversity in various natural environments (Juck *et al.* 2000; Muyzer and Smalla 1998; Smalla *et al.* 2001). This technique separates a mixture of PCR amplicons using the melting temperature distinctive to the DNA sequence of each amplicon (Sheffield *et al.* 1989). In this study, this characteristic of DGGE was used to detect the survival of an introduced bacterium, *Mycobacterium* sp. strain S65, during a bioaugmentation assessment. A DGGE-blot technique using universal 16S rDNA primers was tested for its possible application for the detection of S65 in soil without genetically marking S65.

3.3. Materials and Methods

3.3.1. Soil characteristics

A PAH contaminated soil was obtained from a fire-fighter training area in Trenton, Ontario, Canada. The soil was sampled in December 18, 2000 and was immediately frozen at the site. The soil was then gradually thawed at 4°C for bioaugmentation, chemical, and microbial community analyses. Characteristics of the soil are shown in Table 3.1.

3.3.2. Mineralization assay

Mineralization assays were performed as described in Chapter 2 except that a soil sample was used instead of a cell culture. The biodegradation activity of the indigenous microbial community was tested under eight different conditions: i) no amendment; ii) 1% (wt/wt) alfalfa (hey); iii) 0.1% (v/wt) Tween-80 (non-ionic detergent); iv) 0.1% (wt/wt) commercial fertilizer containing 20% each of nitrogen, phosphorus, and potassium; v) a combination of ii) and iii); vi) a combination of ii) and iv); vii) a combination of iii) and iv); and viii) a combination of ii), iii), and iv). Twenty grams of soil was placed in each microcosm. The control soil microcosms for each condition were prepared by autoclaving twice, with the second autoclaving being performed 24 h after the first autoclaving. The ^{14}C-labelled substrates (Sigma) used in this experiment were [1-^{14}C] hexadecane (specific activity, 2.2 mCi/mmol), [9-^{14}C] phenanthrene (specific activity, 46.9 mCi/mmol), and [4,5,9,10-^{14}C] pyrene (specific activity, 58.7 mCi/mmol); 100,000 dpm of each ^{14}C-labelled substrate was added to each microcosm. The soil microcosms were set up in triplicates and incubated in the dark without agitation for 90 days. Sampling was carried out as described in Chapter 2.

3.3.3. Bacterial strain

Based on the potential to mineralize various PAHs in preliminary experiments,. *Mycobacterium* sp. strain S65 was chosen to remediate pyrene and phenanthrene from the

contaminated soil. S65 was grown in YTS1000 (1g each of yeast extract, tryptone, and starch per l) broth until it reached mid-log phase. The culture was harvested and washed twice with an equal volume of H_2O before inoculating the microcosms.

3.3.4. Bioaugmentation assay

The contaminated soil was bioaugmented with S65 to determine degradation kinetics; six different conditions were tested and are summarized in Table 2. S65 was introduced at two different cell densities: for conditions 2 and 3, S65 was introduced at 10^6 cfu/g of soil; and for conditions 5 and 6, the cell density was 10^7 cfu/g of soil. A fertilizer was added to conditions 3, 4, and 5 to a final concentration of 1% (w/w). Five hundred g of PAH contaminated soil were prepared in triplicate for each treatment; the humidity of the soil was kept above 65% water holding capacity, to maintain optimum microbial activity (Cattaneo *et al.* 1997). Samples were taken periodically at times 0, 3, 7, 14, 21, 36, 56, and 90 days for chemical and microbial community analyses, as described below.

3.3.5. Chemical analysis

Soil samples at time 0 and 90 days were collected to analyze the changes in PAH concentration during bioaugmentation. The soil samples were extracted with 200 ml dichloromethane (DCM) using a Soxhlet extractor as follows: an equal weight of anhydrous sodium sulfate was added to the soil samples and homogenized well using a mortar and pestle. The samples were transferred to thimbles inserted into a Soxhlet extractor and 5 ng p-terphenyl was added as an internal standard. A 500-ml round-bottom flask containing DCM was firmly attached to the extractor to prevent water contamination. Extraction was carried out overnight (16 h) and the resulting extracts were concentrated to 1 ml using a rotary evaporator at 40°C. The extracts were analyzed for the presence of pyrene and phenanthrene by gas chromatography/mass spectrometry (GC/MS). Phenanthrene and pyrene were chosen because S65 is capable of degrading these PAHs.

3.3.6. Bacterial enumeration

Total viable bacterial were enumerated on plates containing 250 mg each of yeast, tryptone, and starch per litre (YTS250) at each sampling time. Aliquots of each sample (ca. 5 g) were diluted in 3 volumes (wt/v) with 0.1% tetrasodium pyrophosphate solution, pH 7.0, vortexed at maximum speed for 2 min, and serially diluted in the tetrasodium pyrophosphate solution. The soil samples and dilutions were always kept on ice. Aliquots (0.1 ml) were plated on YTS250 in triplicate and incubated at room temperature. Colony forming units were counted after 2 weeks of incubation.

3.3.7. Total community DNA extraction

Total community DNA of soil samples was extracted using a method optimized from previously described methods (Fleming *et al.* 1993; Miller *et al.* 1999; Watson and Blackwell 2000). An aliquot (0.75 ml) of soil buffer 1 (50 mM Tris-HCl, pH 8.3, 200 mM NaCl, 5 mM EDTA, and 0.05% Triton X-100) was added to a 2-ml screw-cap polypropylene microcentrifuge tube containing 1:1 (v/wt) of the soil sample. The soil was washed for 30 seconds with mild vortexing and centrifuged at 3,000 X g for 5 min. The supernatant was discarded and an equal volume of soil buffer 2 (50 mM Tris-HCl. pH 8.3, 200 mM NaCl, and 5 mM EDTA) was added to the tube for a second wash. The wash was repeated again with soil buffer 3 (50 mM Tris-HCl, pH 8.3 and 0.1 mM EDTA) and the sample was centrifuged. The sample was resuspended in 675 µl 250 mM Tris buffer (pH 8.0) and 75 µl 20% SDS. Zirconia/silica beads (0.5 g of 0.1 mm diameter) were added to the tube followed by homogenization using a Mini Bead-Beater 8 (Biospec Products) for 2 min. The tube was then incubated at 80°C for 30 minutes with mixing every 10 minutes. The soil lysate was then centrifuged at 16,000 X g for 5 min at room temperature and the supernatant was transferred to a new tube. DNA was extracted twice with an equal volume of phenol/chloroform/iso-amyl alcohol (25:24:1). Residual phenol was removed by extracting twice with chloroform/iso-amyl alcohol (24:1). The crude DNA extract was then precipitated with 1 volume of isopropanol at –20°C overnight. The DNA was centrifuged at 16,000 X g for 20 min at 4°C and washed with 70% ethanol. The crude DNA pellet was dried in a SpeedVac (Savant, Dna101) and dissolved in 100 µl

of TE (10 mM Tris, pH 8.0 and 0.1 mM EDTA). An aliquot (50 µl) was purified using a PVPP spin column (Berthelet *et al.* 1996). The purified DNA was quantified using the PicoGreen dsDNA quantification reagent (Molecular Probes).

3.3.8. Polymerase chain reaction (PCR)

PCR was performed with the total community DNA extracted from conditions 1, 4, 5, and 6 at time 0, 7, 21, 56, and 90 days. PCR was performed to amplify part of the 16S rDNA gene using unversal eubacterial oligonucleotide primers with 5'-*GCGGGCGGGGCGGGGGCACGGGGGGCGCGGCGGCGGGGCGGGGG*CCTAC GGGAGGCAGCAG–3' as the forward primer (Sheffield *et al.* 1989) and 5'-CTACCAGGGTATCTAATCC–3' as the reverse primer (Muyzer *et al.* 1993). A 45-bp GC-clamp was attached to 5' end of the forward primer, indicated in Italics, to increase detection sensitivity of single base-pair differences (Sheffield *et al.* 1989). The PCR mixture contained 0.4 pmol of each primer, 10 mM of each deoxyribonucleoside triphosphate, 2 mM $MgCl_2$, 0.1 to 1 ng of total community DNA, and sterile deionized water to make the a final volume to 50 µl. The tubes were incubated at 96°C for 5 min, cooled to 80°C, at which temperature a *Taq* mixture, 2.5 U of *Taq* polymerase and 5 µl of 10X PCR buffer (10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$), was added to each tube. To increase the specificity of the amplification, a touchdown PCR (Juck *et al.* 2000) in which the annealing temperature was set to decrease 1°C per cycle from 65°C to 55°C, at which temperature 20 additional cycles were carried out. Denaturation was carried out at 96°C for 1 min., the annealing of primers at the appropriate temperature for 1 min., and primer extension was carried out at 72°C for 1 min.

3.3.9. Denaturing Gradient Gel Electrophoresis (DGGE) analysis

PCR products obtained from total community DNA of the bioaugmentation assay were further analyzed for microbial community changes with and without introduction of S65 over time, as well as for the survival of S65 in the presence of the indigenous microbial community. To separate the mixture of 16S rDNA genes into individual 16S

rDNA fragments based on sequence differences, a denaturing gradient gel electrophoresis (DGGE) technique was applied. DGGE was performed using a Bio-Rad Dcode system (Bio-Rad) as previously described (Juck *et al.* 2000). An 8% (wt/v) acrylamide gel containing a linear chemical denaturant gradient ranging from 40 to 70% was prepared by mixing an appropriate volume of a 8% acrylamide-0% denaturant stock solution (8% acrylamide/bisacrylamide (37.5:1) (Bio-Rad) and 1X TAE, 40 mM Tris, 20 mM acetate acid, and 1 mM disodium EDTA, pH 8.0), and a 8%-acrylamide-80% denaturant stock solution (8% acrylamide/bisacrylamide (37.5:1), 1X TAE, 40% deionized formamide, and 7M urea). PCR amplicons were pooled to obtain 370 ng of DNA for treatments 1 and 5 soil samples and 650 ng of DNA for treatments 4 and 6 soil samples and loaded onto the gel. The concentration of DNA was kept consistent for all the samples in the same treatment. The gel was run at 60°C at 80 V for 16 h. After electrophoresis, the gel was stained in a Vistra Green solution and scanned using a FluorImager (Molecular Dynamics).

3.3.10. DGG-blot

A second DGGE containing exactly the same samples as above, was prepared for hybridization. The gel, eight pieces of Whatman paper precut to the size of the gel, and a Zeta-Probe membrane (Bio-Rad) were soaked in 0.5X TBE for 15 – 30 min prior to electrophoretic transfer. Electrophoretic transfer of DNA from the acrylamide gel to the membrane was carried out using a Trans-Blot SD (Bio-Rad) apparatus, according to the manufacturer's instructions. First, four pieces of pre-soaked paper were placed onto the platinum anode and rolled over using a test tube to remove any air bubbles. The pre-soaked membrane was placed with the gel on the top of the membrane, followed by placing four pieces of paper on top of the membrane. The transfer was carried out at 400 mA for 1.5 h. After the transfer, DNA was fixed to the membrane a using UV crosslinker (Strategene). The membrane was then rinsed briefly in 0.5X TBE buffer, followed by soaking in 0.4N NaOH and 2X SSC each for 10 min. The membrane was then placed between pieces of blotting paper and stored at room temperature until hybridization was performed.

Hybridization was carried out as described in the Southern hybridization of Chapter 2. A PCR fragment of a partial S65 16S rDNA amplified with the same primers as for the environmental samples was used as a probe as well as a positive control for hybridization. The hybridization temperature was set at 65°C to increase stringency of the detection.

3.4. Results

3.4.1. Soil chemistry

The contaminated soil was tested for the level of PAH contamination. The chemical analysis of the soil revealed that the contamination level was below the Ontario provincial criteria for contaminated soils (Table 1.2) (Environment Ontario 1992).

3.4.2. Mineralization potential of indigenous microorganisms

The biodegradation ability of the indigenous microbial community for hexadecane, phenanthrene, and pyrene was examined in microcosms at room temperature for 90 days (Figure 3.1). Hexadecane was effectively mineralized when the soil was amended with the fertilizer (69% mineralization) and the combination of the fertilizer and Tween-80 (68% mineralization). When Tween-80 was added alone the mineralization extent (40% mineralization) was below the extent (47% mineralization) for the reference (no amendments). When no amendments were added to microcosms, a lag phase in hexane mineralization was observed.

When Tween-80 and the fertilizer were added individually to the soil, the best degradation of phenanthrene was observed (58% mineralization for both). Alfalfa was a poor amendment for phenanthrene degradation. Pyrene was not mineralized to the extent as other substrates tested in this assay. The maximum mineralization extent (18.7%) was achieved when Tween-80 and alfalfa were added as amendments.

3.4.3. PAH analysis of soils bioaugmented with S65

The effect of different treatments for PAH remediation were studied by comparing the changes in phenanthrene and pyrene concentrations at day 0 and day 90 during the bioaugmentation assay. The colour of the total community DNA extracts varied depending on the treatments. The extracts from the treatments with fertilizer and inoculum (treatment 3, 4, and 5) gave the darkest colour, whereas the extracts from the control treatment (treatment 1) gave a much lighter brown colour. The chemical analysis indicated that the concentrations of phenanthrene and pyrene varied in all the treatments at T=0 when the concentrations should have been similar (Table 3.3). The analysis also showed that remediation of these PAHs was not enhanced by the introduction of S65 or the fertilizer (20:20:20) in thissoil. No significant loss of phenanthrene was observed in most treatments except in treatment 1 and treatment 3, in which half of the original concentration of phenanthrene was degraded. The same phenomena were observed for pyrene remediation. The concentration of pyrene was greatly reduced after 90 days in treatment 1. No significant loss of pyrene was observed in treatments bioaugmented with S65, fertilizer, or combinations of both.

3.4.4. Viable microbial population

Viable microbial populations in different bioaugmentation treatments were determined by plate counts. The soil samples were taken a total of 8 times throughout the study and plated on YTS250 plates in triplicate. An increase in microbial population was observed in all treatments (Figure 3.2). A significant increase (~ 10-fold) in microbial biomass was observed when the fertilizer was added as an amendment. The total viable counts for treatments 3 and 5 were similar although 10^7 cfu/g of S65 was seeded in treatment 5 whereas 10^6 cfu/g of S65 was seeded in treatment 3. The total population size for treatments 2 and 6 was similar to the control treatment, indicating that introducing cells at 10^{6-7} cfu/g had no effect on the overall population size of the treatments. When fertilizer was added to treatments, the total viable counts increased 10 fold more than the control treatment after 90 days independent of the size of the introduced cells.

3.4.5. Optimization of soil DNA extraction

Total community DNA was extracted using a bead-beating method modified to enhance the quality and quantity of DNA extracts. Usually, bead-beating methods shear DNA, but this problem was resolved by reducing the speed and time of the bead-beating process. All the crude DNA extracts were brown, an indication of the presence of humic acids. A dark brown colour was observed in the extracts from the soil samples in the fertilizer treatments. The colour of extracts became darker following longer incubation times (data not shown). All the DNA extracts were purified using PVPP spin columns to remove humic acids. Most of the extracts became clear after the purification step except those in treatments 4 and 5, which needed to be PVPP-purified twice. The quality of DNA extracts was assessed by agarose gel electrophoresis to specifically look for a large genomic DNA band with little sheared lower molecular weight material. The DNA extracts from treatment 1 are shown in Figure 3.3. Total community DNA at the 23 Kb level was produced in each extract, indicating that the quality of soil DNA had not been lowered by the bead-beating process.

3.4.6. Bacterial community analysis using DGGE

Bacterial communities from the different treatments were compared using a PCR-DGGE technique. Partial 16S rDNA fragments were amplified from the DNA extracts of treatments 1, 4, 5, and 6 at 0-, 7-, 21-, 56-, and 90 days. These days were selected because no significant changes were observed in other time intervals in the viable microbial counts. The concentration of denaturants in the gels was set between 40–70% for a 16h electrophoresis but it was later discovered that 40–65% denaturants gave a better result. Therefore, DNA samples for treatments 1 and 5 were loaded onto a 40–70% denaturing gel (Figure 3.4A), whereas those for treatments 4 and 6 were loaded onto a 40–65% denaturing gel (Figure 3.4B). The bacterial diversities, estimated using the number of bands, and the bacterial populations, estimated by the intensity of the bands, were compared among the treatments. The proportional abundance of the population was possible because the concentration of DNA for each sample was normalized prior to the DGGE application. The DGGE profiles showed that the microbial community structures

were already different at time 0 in all the treatments except treatments 4 and 6 which had a similar community pattern (Figure 3.4A and B). In treatment 1, no significant structural changes were observed throughout the bioaugmentation study; the relative abundance of each band and the diversity of bacteria were unchanged. In other treatments, fluctuations in population sizes of some bacteria and diversity were clearly observed. In both treatments 5 and 6, the band corresponding to S65 was not detected, even at time 0.

3.4.7. DGG-blot

DNA on the denaturing gels was blotted onto membranes for the detection of S65 by hybridization using a 16S rDNA PCR amplicon, produced with S65 genomic DNA. The time for the electrotransfer was extended to 1.5h instead of the recommended time (10 min) (Bio-Rad) because acrylamide gels were used instead of agarose gels. Acrylamide gels have tighter matrices, making it more difficult for DNA to diffuse. The hybridization result from the membrane containing DNA samples of treatments 4 and 6 showed that multiple signals were detected (Figure 3.5). The S65 16S rDNA PCR amplicon was also detected by hybridization. Most of the detected signals in the treatments were observed in positions in the membrane above the S65 16S rDNA PCR amplicon signal. .

3.5. Discussion

Bioaugmentation is a method to remediate pollutants in the environment by introducing organisms, which are capable of degrading the pollutants of interest. Application of microorganisms to remediate contaminants is of considerable interest in a social context because it is environmental friendly and cost effective (Head 1998). In this study, *Mycobacterium* sp. strain S65 was tested for phenanthrene and pyrene remediation in a PAH contaminated soil since this strain had demonstrated its ability to degrade phenanthrene and pyrene to a great extent in laboratory microcosms (Chapter 2). Results from the chemical analysis of PAHs (phenanthrene and pyrene) showed that the concentrations of phenanthrene and pyrene were below the Canadian regulatory criteria (40 mg/kg for phenanthrene and 250 mg/kg for pyrene) before the bioaugmentation study

started. No significant decrease in concentration of both PAHs was observed during bioaugmentation. This could be because there was not enough phenanthrene and pyrene in the soils to stimulate degradation by S65. Uneven spatial distribution of an inoculum during a bioaugmentation study has been reported by Boon *et al.* (2000). When the green fluorescent protein (gfp) tagged bacterium, *Comamonas testosteroni* strain I2 was visibly detected in an activated sludge as green cells under UV light, it formed a cluster within a sludge floc. It could be possible that S65 inoculum was not distributed evenly and the PAHs were not bioavailable to S65.

During the bioaugmentation study, the phenanthrene concentration detected increased slightly in treatments 5 and 6. In treatment 4, the detected concentrations of both PAHs also increased. This increase could be explained by the increase in extractable PAHs during the study, or may indicate heterogeneity in the soil. The influence of surfactants on solubility and desorption rates of PAHs is well known (Barkay *et al.* 1999; Thibault *et al.* 1996; Tiehm 1994; Volkering *et al.* 1995). It is possible that the addition of fertilizer and the inoculum had an impact on the bioavailability of these PAHs. Variability of PAH concentrations was also observed from samples in all treatments during the study. At time 0, the concentration of phenanthrene ranged from 16 mg/kg in treatment 2 to 6.5 mg/kg in treatment 6. In the case of pyrene, it ranged from 28 mg/kg in treatment 1 to 10 mg/kg in treatment 4. This inconsistency in the PAH concentrations in the samples shows a heterogeneity of the soil samples (Harms and Bosma 1997), despite being well mixed before sampling.

Total viable counts of bacteria demonstrated an increase in the microbial population in all treatments including the control treatment (treatment 1). A one-fold increase in total counts was observed in the control treatment. No amendments were applied in this treatment. The possible reasons for this increase are the increased soil aeration and maintaining of the water holding capacity above 65% throughout the study. The high moisture and oxygen levels would influence microbial growth in the control soil (Cattaneo *et al.* 1997; Wilson and Jones 1993). Fertilizer also had a great impact on microbial growth. Interestingly, the inoculation of S65 alone had no effect on the soil microbial population size. The total counts in treatment 5 were higher than those of treatment 2 at time 0 but the counts became the same in both treatments 7 days after

inoculation. Of interest, the microbial population sizes of treatments 2 and 5 were more or less the same as that of treatment 1. A decrease in the population size of the inoculum is a common phenomenon (van Veen *et al.* 1997). When *Comammonas testosteroni* strain I2 was reintroduced into an activated sludge containing 3-chloroaniline, from which the inoculum was isolated, to enhance degradation of 3-chloroaniline, the population size of I2 decreased by one fold 30 days after inoculation (Boon *et al.* 2000). S65 might not be able to compete effectively against indigenous microorganisms for space and nutrients.

Efficient extraction of DNA from soil samples plays an important role in molecular analyses. High molecular weight (HMW) DNA needs to be recovered from the soil to avoid chimera formation during PCR amplification. The technique used in this study to extract total community DNA from soils was optimized from trials of different extraction techniques previously published (Flemming *et al.* 1994; Fortin *et al.* 1998; Miller *et al.* 1999; Purdy *et al.* 1996). A physical lysis method (bead beater) was chosen because the cell wall of *Mycobacterium* sp. strain S65 is thick and it has been reported that Gram-positive and spore-forming bacteria readily survive other types of lysis treatment such as a freeze-thaw and enzymatic treatment (Kuske *et al.* 1998). It was also observed during the preparation of genomic DNA from S65 that the DNA was not released from the cells after a hot SDS treatment unless the cells had been treated previously by bead-beating (data not shown). As seen in Figure 2, no shearing of HMW DNA was observed in all soil samples using a bead homogenization technique. A combination of the bead beating treatment and lysozyme treatment was also tested but there was no significant difference in the quality and quantity of total DNA extracted (data not shown).

Mineralization results for the indigenous microbial community showed that the microorganisms in the community were capable of degrading pyrene. PCR amplification, using primers from the pyrene large subunit dioxygenase gene, *nidA*, was carried out to assess for the presence of the *nidA* gene in the indigenous soil microbial population. A positive amplification of the *nidA* gene (data not shown) indicated the presence of microorganisms possessing the *nidA* gene, therefore, the *nidA* gene was not used as a primer for the detection of S65 because it would be difficult to distinguish S65 from pyrene degrading indigenous bacteria in the soil. As an alternative, a variable region of

16S rDNA, which produced a 418 nt long PCR amplicon, was used for the detection of S65. In this study, the DGG-blot technique was used to detect the survival of S65 in soil, using the 16S rDNA PCR amplicon of S65 as a probe. The DGG-blot technique was first developed for the detection of DNA sequence polymorphisms in human genomic DNA (Gray 1992). Optimization of the blotting protocol was necessary because the apparatus (Trans-Blot SD DNA/RNA Blotting Kit, Bio-Rad) used for electrotransferring DNA from an acrylamide gel onto a membrane only gave instructions for DNA transfer from an agarose gel. Considering that an acrylamide gel has a tighter matrix than an agarose gel, the duration of electrotransfer was set at 1.5 h instead of 10 min. In the original paper (Gray 1992), the electrotransfer step was performed for 2 h at 30 – 80 V at 55°C. In this study, a current of 400 mA at 25 V was used, as recommended by a manufacturer.

The DNA hybridization result from treatments 4 and 6 detected multiple bands using the S65 16S rDNA PCR probe. Because sequences of 16S rDNA genes are very common in the members of mycobacteria, high similarity (> 98%) was obtained from other mycobacteria and only a few base pair differences were observed between S65 16S rDNA gene sequence to others. Because of the high similarity of the sequences, the bands detected in the hybridization could represent other mycobacteria present in the soil.

The survival and ability of *Mycobacterium* sp. strain S65 to act as a bioaugmentation agent for PAH remediation in contaminated soil was examined in this study. Difficulties were encountered optimizing the PAH remediating potential of S65. Boon *et al.* (2000) overcame this problem by reintroducing the inoculum during the bioaugmentation study. The use of a 16S rDNA probe was not found to be appropriate to specifically detect S65 in the soil. A few bp differences over the 418 bp length of the probe (the mismatches were scattered throughout the sequences) (data not shown), did not give a sufficiently high specificity. Other genes are recommended for use to detecta strain of interest. The results from this study suggested that S65 did not survive well or remain particularly active in the contaminated soil as indicated by the poor PAH degradation observed under all test conditions. This study shows there is still a need of an optimisation for efficient bioremediation.

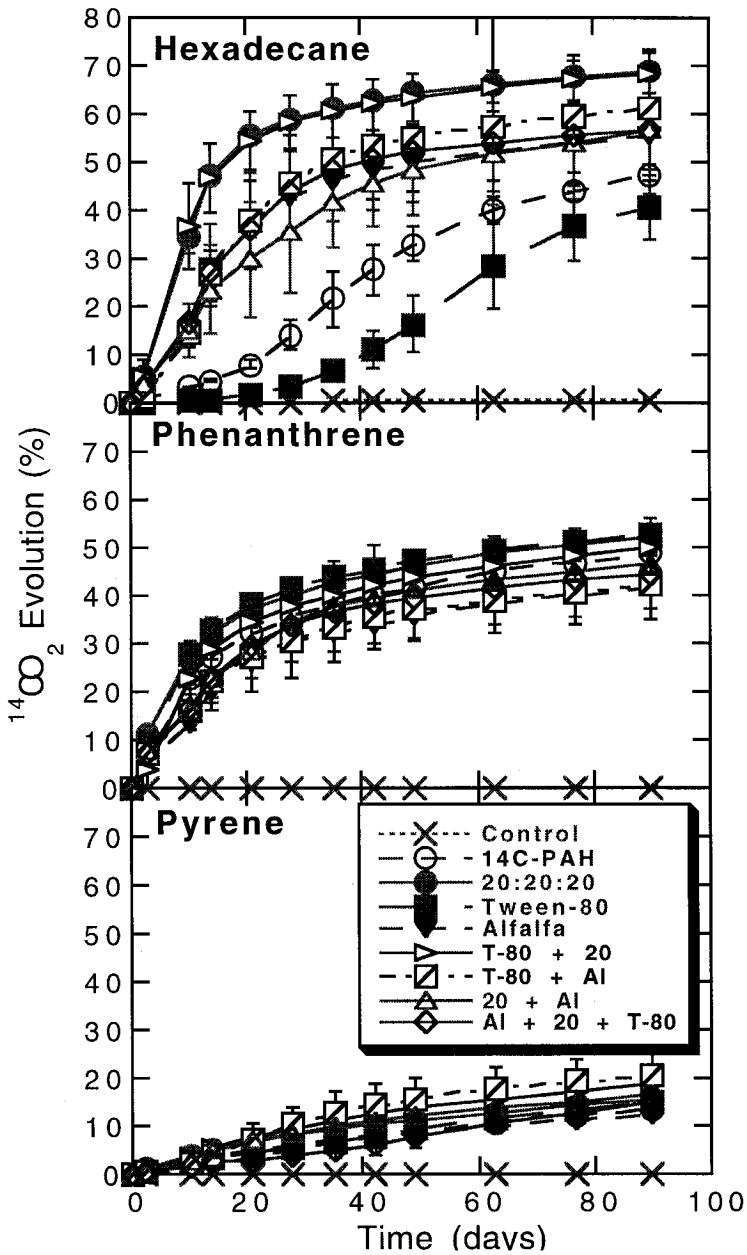


Figure 3.1. Mineralization of hexadecane, phenanthrene, and pyrene by the indigenous microbial population in soil microcosms. The PAH contaminated soil was tested for hexadecane (A), phenanthrene (B), and pyrene (C) mineralization under various bioaugmentation conditions. The controls for each condition were made with heat-killed soils.

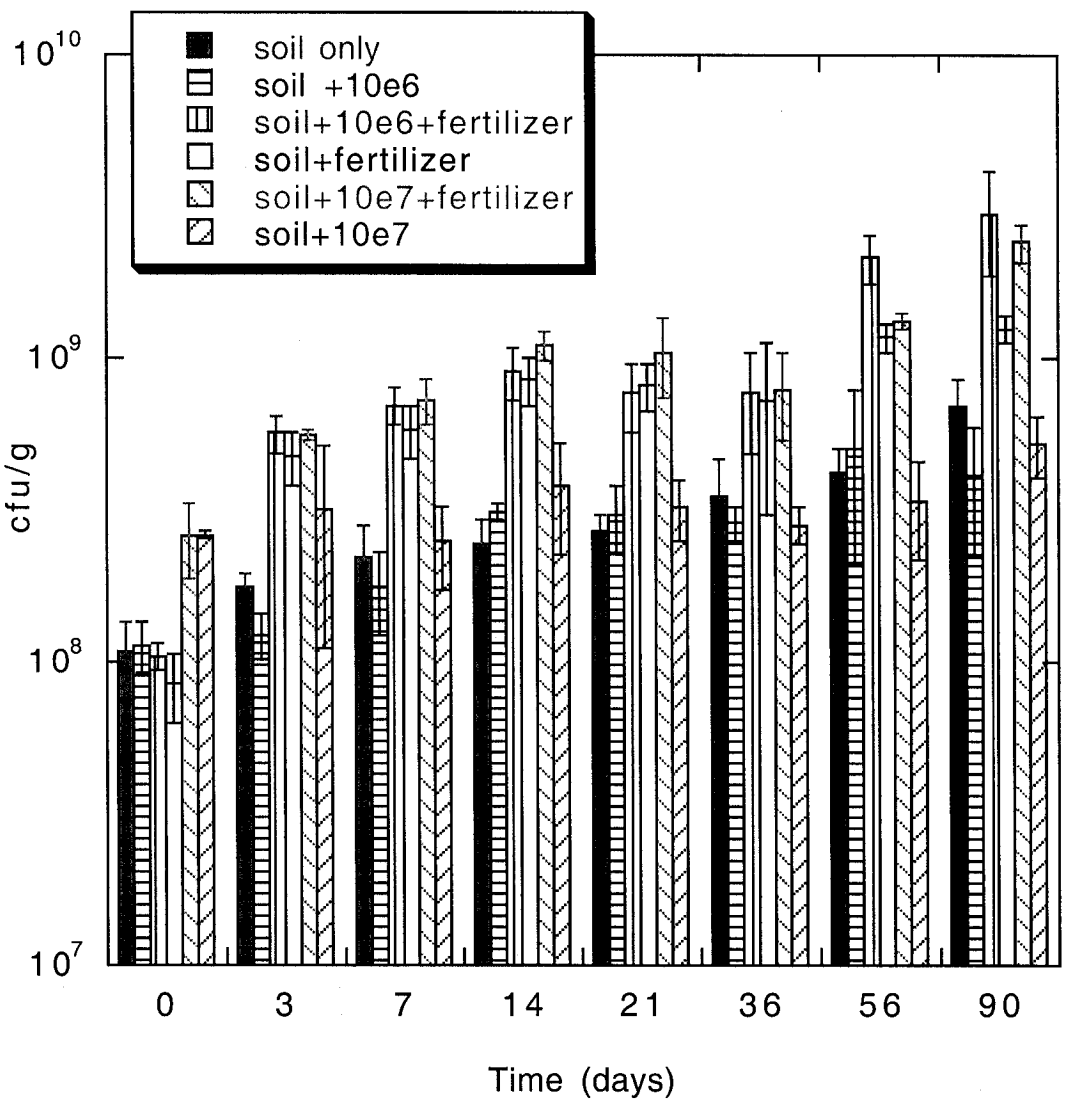


Figure 3.2. Enumeration of viable bacteria in the bioaugmented soil over the 90 day treatment period.

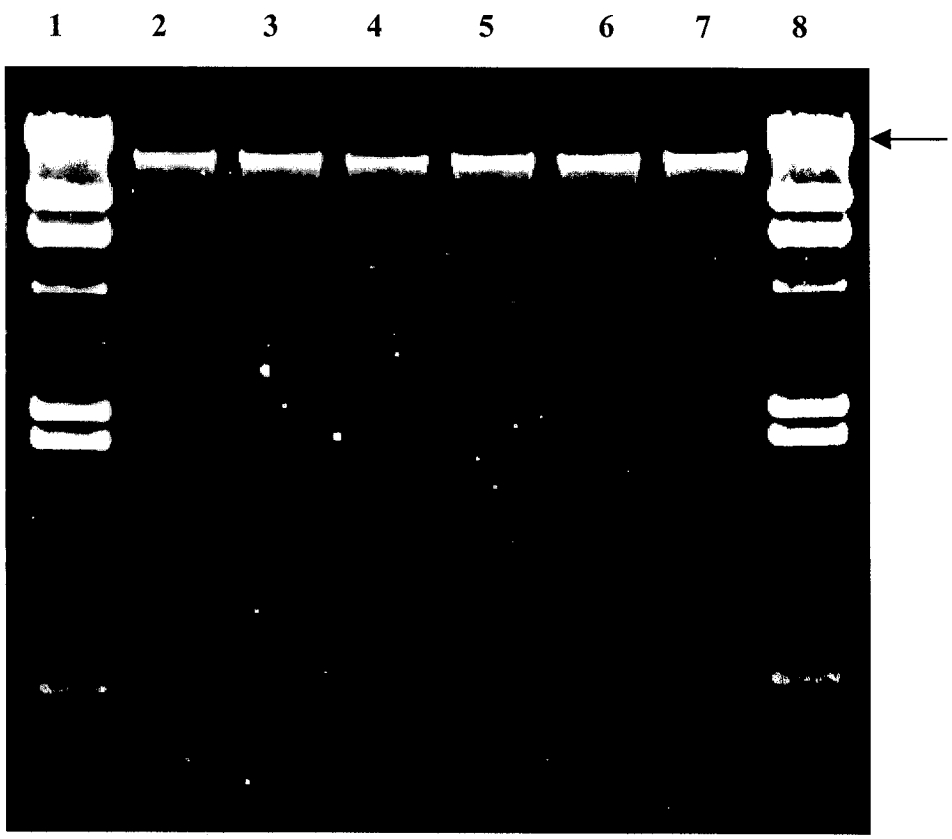


Figure 3.3. Total community DNA from the PAH contaminated soil. This figure shows an example of how the total community DNA extracts look on agarose gels. Lanes 1 and 8 represent a λ DNA digested with *Hind*III marker; lanes 2 through 4 represent the DNA extracts of triplicate samples at T=21. Lanes 5 through 7 represent the DNA extracts of triplicate samples at T=36. All the samples are from treatment 5. The arrow indicates a 23 Kb molecular weight.

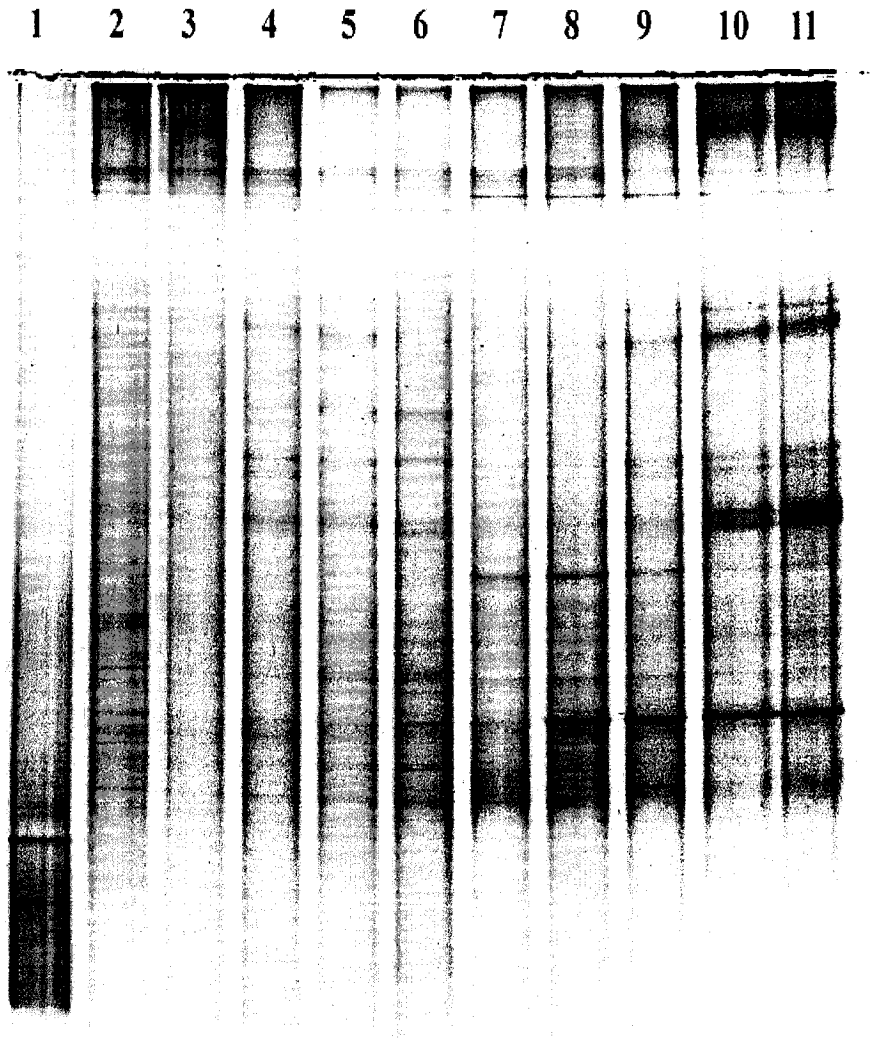


Figure 3.4A. DGGE profile of the partial 16S rDNA fragments in treatments 1 and 5 during bioaugmentation. Lanes 2 to 6 represent the community profiles in treatment 1 with lane 2, T=0; lane 3, T=7; lane 4, T=21, lane 5, T=56, and lane 6, T=90. Lanes 7 to 11 represent the community profiles in treatment 5 with lane 7, T=0; lane 8, T=7; lane 9, T=21, lane 10, T=56, and lane 11, T=90. Lane 1 represents the S65 positive control. The denaturant gradient was made between 40 – 70%.

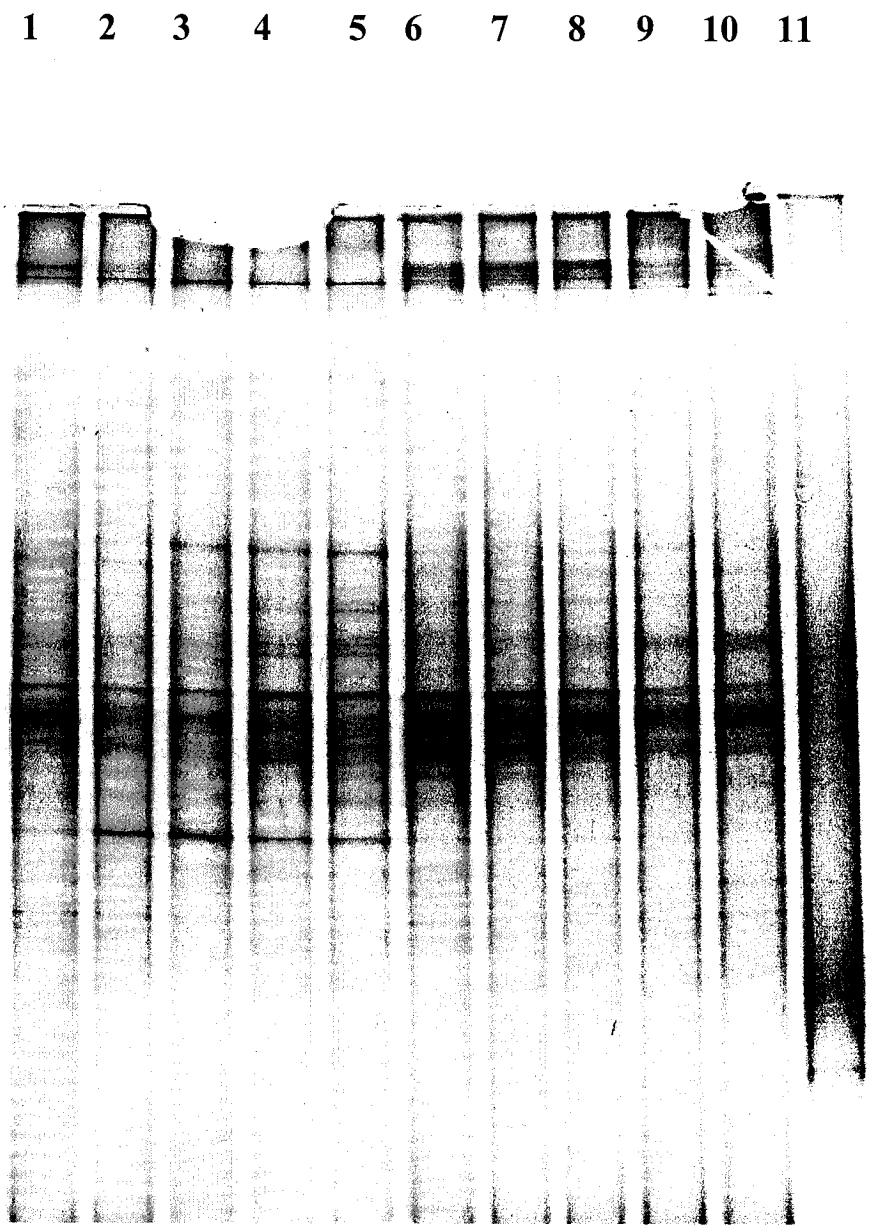


Figure 3.4B. DGGE profile of the partial 16S rDNA fragments in treatments 1 and 5 during bioaugmentation. Lanes 1 to 5 represent the community profiles in treatment 4 with lane 1, T=0; lane 2, T=7; lane 3, T=21, lane 4, T=56, and lane 5, T=90. Lanes 6 to 10 represent the community profiles in treatment 6 with lane 6, T=0; lane 7, T=7; lane 8, T=21, lane 9, T=56, and lane 10, T=90. Lane 11 represents the S65 positive control. The denaturant gradient was made between 40 – 65%.

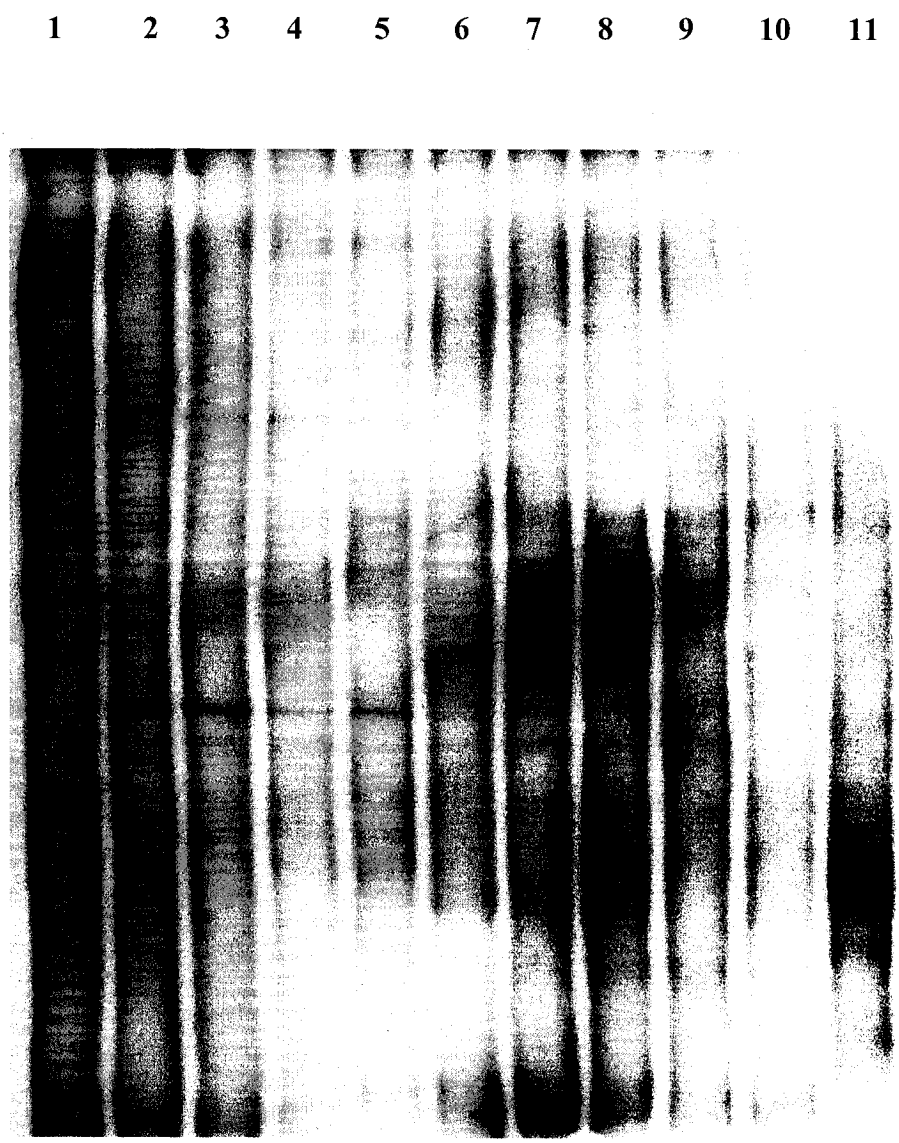


Figure 3.5. Hybridization of denaturing gradient gel with the PCR amplicon of S65 partial 16S rDNA as a probe. Lanes 1 to 5 represent the samples from treatment 4 and lanes 6 to 10 represent the samples from treatment 6. Lanes 1 and 6, T=0; lanes 2 and 7, T=7; lanes 3 and 8, T=21; lanes 4 and 9, T=56; lanes 5 and 10, T=90; lane 11, PCR amplicon of S65 partial 16S rDNA

Table 3.1. Physico-chemical characteristics of Trenton soil.

Soil Analyses	
% humidity	18
Total phosphate (mg/kg)	720
Total nitrogen (mg/kg)	640

Table 3.2. Bioaugmentation treatments

Treatment	Bioaugmentation		
	fertilizer (20:20:20)	S65	
		10^6 cfu/g	10^7 cfu/g
1	-	-	-
2	-	+	-
3	+	+	-
4	+	-	-
5	+	-	+
6	-	-	+

Table 3.3. PAH analysis of the soil bioaugmented with S65

	PAHs (mg/kg) average in triplicate samples			
Soil condition	Phenanthrene		Pyrene	
	0 (day)	90	0 (day)	90
#1	11.5	4.5	28	10
#2	16	11	24.5	17.5
#3	15	6.7	21	12.3
#4	7.5	7.7	10	13.7
#5	7	7.3	16	14
#6	6.5	7	18.5	11.5

4. Conclusions

Biodegradation of PAHs is a relatively new field of study. The genetic and biochemical pathways of LMW-PAHs have been well documented, but the degradation pathways of many other PAHs, especially HMW-PAHs, have not yet been elucidated. A lack of bacterial isolates which are capable of degrading HMW-PAHs and difficulty in manipulating the genes involved in the HMW-PAH degradation are some of the factors limiting the amount of knowledge in this area. Since the discovery of pyrene catabolic genes in *Mycobacterium* sp. strain PYR-1 in combination with the elucidation of pyrene catabolic metabolites, characterization of pyrene degradation at the genetic and biochemical levels has been greatly facilitated. In this study, some of the putative genes involved in pyrene catabolism in *Mycobacterium* sp. strain S65 were isolated and characterized. A new upper pathway involved in pyrene degradation was identified in strain S65 that appeared to be induced by pyrene. This information is crucial to our understanding of the assimilation pathways of HMW-PAHs by bacteria and may allow us to investigate the occurrence and distribution of PAH degradation genes and PAH degradation pathways other than the current known PAH catabolic pathway from the *nah* (or *nah*–like) genes.

Determining the factors that limit PAH biodegradation in the environment is also important for success in bioremediation. Bacteria, which are very efficient degraders of HMW-PAHs in a laboratory setting, do not necessary behave the same way when they are re-introduced into natural environments. There are a lot of factors, i.e., understanding the behaviour of introduced bacteria, the interaction between the introduced bacteria and indigenous bacteria, and bioavailability of nutrient sources including PAHs, that need to be optimized. Using molecular analyses, such as amplifying a specific gene by PCR can enhance the limit of detection of organisms or certain genes present in the environment compared to using traditional plating techniques. By DGGE in combination of DGG-blot, a diversity of genes of interest can be determined as well as the dynamics of the gene within the microbial population diversity.

Appendices

Appendix1-1. Sequence of 16S rDNA of *Mycobacterium* sp. strain S65

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1     ATGCAAGTCG AACGGTAAGG CCCTTCGGGG TACACGAGTG CGAACGGGTG
51    AGTAACACGT GGGTGATCTG CCCTGCACTT TGGGATAAGC CTGGGAAACT
101   GGGTCTAATA CCGAATATGA TCATGGCCTT CATGGGTTGT GGTGGAAAGC
151   TTTTGCGGTG TGGGATGGGC CCGCGGCCTA TCAGCTTGTT GGTGGGGTAA
201   TGGCCTACCA AGGCGACGAC GGGTAGCCGG CCTGAGAGGG TGTCCGGCCA
251   CACTGGGACT GAGATACGGC CCAGACTCCT ACGGGAGGCA GCAGTGGGGA
301   ATATTGCACA ATGGGCGCAA GCCTGATGCA GCGACGCCGC GTGAGGGATG
351   ACGGCCTTCG GGTTGTAAAC CTCTTTCAGT AGGGACGAAG CGCAAGTGAC
401   GGTACCTATA GAAGAAGGAC CGGCCAACTA CGTGCCAGCA GCCGCGGTAA
451   TACGTAGGGT CCGAGCGTTG TCCGGAATTA CTGGGCGTAA AGAGCTCGTA
501   GGTGGTTTGT CGCGTTGTTC GTGAAAACTC ACAGCTTAAC TGTGGGCGTG
551   CGGGCGATAC GGGCAGACTG GAGTACTGCA GGGGAGACTG GAATTCCTGG
601   TGTAGCGGTG GAATGCGCAG ATATCAGGAG GAACACCGGT GGCGAAGGCG
651   GGTCTCTGGG CAGTAACTGA CGCTGAGGAG CGAAAGCGTG GGGAGCGAAC
701   AGGATTAGAT ACCCTGGTAG TCCACGCCGT AAACGGTGGG TACTAGGTGT
801   GGGTTTCCTT CCTTGGGATC CGTGCCGTAG CTAACGCATT AAGTACCCCG
851   CCTGGGGAGT ACGGCCGCAA GGCTAAAACT CAAAGAAATT GACGGGGGCC
901   CGCACAAGCG GCGGAGCATG TGGATTAATT CGATGCAACG CGAAGAACCT
951   TACCTGGGTT TGACATGCAC AGGACGTGCC TAGAGATAGG TATTCCCTTG
1001  TGGCCTGTGT GCAGGTGGTG CATGGCTGTC GTCAGCTCGT GTCGTGAGAT
1051  GTTGGGTTAA GTCCCGCAAC GAGCGCAACC CTTATCTTAT GTTGCCAGCG
1101  CGTAATGGCG GGGACTCGTG AGAGACTGCC GGGGTCAACT CGGAGGAAGG
1151  TGGGGATGAC GTCAAGTCAT CATGCCCCTT ATGTCCAGGG CTTCACACAT
1201  GCTACAATGG CCGGTACAAA GGGCTGCGAT GCCGTGAGGT GGAGCGAATC
1251  CTTTCAAAGC CGGTCTCAGT TCGGATCGGG GTCTGCAACT CGACCCCGTG
1301  AAGTCGGAGT CGCTAGTAAT CGCAGATCAG CAACGCTGCG GTGAATACGT
1351  TCCCGGGCCT TGTACACACC GCCCGTCACG TCATGAAAGT CGGTAACACC
1401  CGAAGCCGGT GGCCTAACCC CTTGTGGGAG GGAGCCGTCG AAGGTGGGAT
1451  CGGCGATTGG GACGAAGTCG TAAC
```

Appendix 1-2. Identification of strain S65 by comparing 16S rDNA sequences in GenBank[a]

	E value[b]
Mycobacterium sp. T104 ...	0.0
Mycobacterium sp. T103 ...	0.0
Mycobacterium sp. RJGll.135...	0.0
Mycobacterium rhodesiae...	0.0
Mycobacterium alvei ...	0.0
Mycobacterium sp. 16S rRNA...	0.0
Mycobacterium sp. 16S rRNA...	0.0
Mycobacterium parafortuitum	0.0
Mycobacterium farcinogenes.	0.0
Mycobacterium fortuitum	0.0
Mycobacterium holsaticum	0.0
Mycobacterium chlorophenolicus (PCP-1)	0.0
Mycobacterium komossense	0.0
Mycobacterium ratisbonense	0.0
Uncultured bacterium clone	0.0
Mycobacterium sp. M0183 1	0.0
Mycobacterium fortuitum	0.0
Mycobacterium frederiksbergense	0.0
Mycobacterium anthracenicum	0.0
Mycobacterium aurum	0.0
Mycobacterium sp. gene	0.0
Mycobacterium ratisbonense.	0.0
Mycobacterium sp. partial sequence	0.0
Mycobacterium sphagni.	0.0
Mycobacterium aichiense	0.0
Mycobacterium sp. SM7.6.1.	0.0
Mycobacterium sp. 'Fuerth 1999'	0.0
Mycobacterium fortuitum...	0.0
Mycobacterium sp. 16S rRNA...	0.0
Mycobacterium sp. PYR-I ...	0.0

[a] Bacterial species are listed in an order of highest sequence similarity to the sequence of S65 by BlastN.

[b] A parameter that describes the number of hits one can "expect" to see just by chance when searching a database of a particular size.

The results shown above were obtained from the BlastN search using the 1474-bp partial 16S rDNA sequence of S65 as a query.

Appendix 1-3. Physiological properties of strain S65 using Biolog GP2 Microplate

Characteristic	Reaction	Characteristic	Reaction
Utilization of:			
L-arabinose	-	L-fucose	-
α-D-lactose	-	D-mannose	+
β-methyl D-glucose	-	L-rhamnose	+
D-tagatose	-	Acetic acid	+
Lactamide	-	Methyl pyruvate	+
Alaninamide	-	L-glutamic acid	-
Adenosine	-	Adenosine-5'-monophosphate	-
α-cyclodextrin	-	Mannan	-
D-arabitol	-	D-galactose	-
Lactulose	-	D-melezitose	-
α-methyl D-mannoside	-	D-ribose	-
D-trehalose	+	α-hydroxybutyric acid	+
D-lactic acid methylester	+	Monomethyl succinate	+
D-alanine	-	Glycyl-L-glutamic acid	-
2-deoxyadenosine	+	Thymidine-5'-monophosphate	-
β-cyclodextrin	-	Tween-40	+
Arbutin	-	D-galacturonic acid	-
Maltose	+	D-melibiose	-
Palatinose	-	Salicin	-
Turanose	+	β-hydroxybutyric acid	+
L-lactic acid	+	Propionic aicd	+
L-alanine	+	L-pyroglutamic acid	+
Inosine	-	Uridine-5'-monophosphate	-
Dextrin	-	Tween-80	+
Cellobiose	-	Gentiobiose	-
Maltotriose	-	α-methyl D-galactoside	-
D-psicose	+	Sedoheptulosan	-
Xylitol	-	γ-hydroxybutyric acid	-
D-malic acid	+	Pyruvic acid	+
L-ananylglycine	-	L-serine	+
Thymidine	-	Fuctose-6-phosphate	-
Glycogen	-	N-acetyl-D-glucosamine	+
D-fructose	+	D-gluconic acid	+
D-mannitol	+	β-methyl D-galactoside	-
D-raffinose	-	D-sorbitol	-
D-xylose	+	p-hydroxyphenyl acelic	-
L-malic acid	+	Succinamic acid	+
L-asparagine	-	Putrescine	+
Uridine	-	Glucose-1-phosphate	-
Inulin	-	N-acetyl-D-mannosamine	-

Characteristic	Reaction	Characteristic	Reaction
α-D-glucose	+	Amygdalin	-
3-methyl glucose	-	m-inositol	-
Stachrose	-	α-methyl D-glucoside	-
α-ketoglutaric acid	-	Sucrose	-
Succinic acid	-	α-ketovaleric acid	-
2,3-butanediol	-	N-acetyl L-glutamic acid	-
Glucose-6-phosphate	-	Glycerol	-
D-L-α-glycerol phsphate	-		

Appendix 1-4 Complete nucleotide sequence of the *nid* locus (GenBank accession number: AF546904)

```
1     CCGATTCCAG AAAGGGTCCA ACCATATGAC CACCGAAACA ACCGGAACAG
                                 nidA
51    CTGACGCGAC CGATCCCTAC CTGCGGCGCG CGTTGCGGGA GGTGGCGGAC
101   GGGCTCAAGG TCGGGCGCTT ACCGGCCCGC GTCGTCAGCG ATCCCGCGCT
151   ACACACGATC GAGATGGAGC GGATCTTCGG GCGCGCCTGG GTGTTTCTCG
201   GACACGAGTC GGAGTTGGCC AAGTCCGGCG ACTTCGTCGT GCGGCACATC
251   GGGGCCGATT CGGTGATCGT TTGCCGGGAC AACTCCGGCC GCATCCAGGC
301   GCTGTCCAAT TCTTGTCGCC ACCGTGGTGC GCTCGTGTGC CGCGCTGAGA
351   TGGGAAACAC CGCGCACTTC CAATGCCCGT ACCACGGCTG GGTGTACAGC
401   AACACCGGAG AGCTCGTCGG CGTGCCGGCG ATGAGTGAGG CCTATCCCGG
451   CGGCTTCGAC AAGTCGCAGT GGGGATTACG TCACATCCCC CATGTCGACT
501   CGTACGCCGG ATTCATCTTC GGCAGCGTCG ATCCGAAGGC GCCGAGCCTG
551   ACCGACTACC TCGGCGACAC GACGTTCTAC CTCGACCTCA TTGCGAAGAA
601   GACAGCGGGC GGTCTGGAGG TGATAGGGGC ACCGCATCGA TGGGTGATGT
651   CAGCGAACTG GAAGACAGCC GCCGACAATT TTGTCGGCGA CTCCTACCAC
701   ACCCTCTTTG CTCACCGCTC GATGGTCGAG CTAGGCATGG CGCCCGGTGA
751   CCCAAACTTC GCGAGCGCAC CAGCGGAGAT CTCGCTGCAG AACGGCCACG
801   GCGTCGGCGT ACTCGGCTTT CCGCCCACGC TCGCCGATTT TCCCGAGTAC
851   GAGGGATACC CCGACGAAGT CGTCGACCAG ATGGCGACGT CCTACCCGTC
901   GCCGGTACAC AAGGACCTGA TGCGACGCTC AGCCTTTATT CACGGCACCG
951   TGTTCCCGAA TTTGTCGTTC ATCAACGTGA CCATCGCGCC GGACCACATG
1001  TCGCCCCCTA CCCCCTTCAT CACGTTCCGG GTATGGCATC CGCTCTCCCA
1051  TGATCGGATG GAGATCCTCT CCTGGTTCCT GGTCGAACGC GATGCTCCGG
1101  AATGGTTGCG CGATGCGTCC CAGGCGTCCT ACGTCAACAA CTTCGGCCCA
1151  GGTGGGGTTT TCGAACAGGA CGACGCCGAG GCATGGAAGG CCATCACCGA
1201  ATCTGTGCAG GGCCCGTTCG CCGGTGCAGG CCTGCTGAAC TACGAAATGG
1251  GCATGGACTT GACTCCGCTC ACCGACTGGC CAGGGCCGGG AGAGGCCCTC
1301  CCGAGCGGGT ACGCCGAGCA GAATCAGCGG CGGTTTTGGG GGAGATGGCT
1351  GGAATACATG GGTCAGCCTC CCGCATTCGG CGGGCGTGCT TGATGAAGCT
1401  TCTGCCCAGG CTGTGCGCGA AGTGCGCCCG TTCACCAATC GGCGCCGCGT
1451  GAGTGCCGTG AAGGCGGGGG ACCGCCGGGT CGCAATTGTC ACTGCCGCCG
```

1501 CCGCGGGAAT CGGTAAGGCG ATCGCCCTGC GGTGGTGCCG CGAAGGTGGG

1551 TGCTGTGTC**A TG**GCCGACAC CGACGGCGAG GGCCTCGATG CGGCCGTGGC

ADH-short

1601 CGAATTGGCC GAGTCCGGCG CGGCGGTTTG CGGCGTCGCC GGCGACGTTT

1651 GCCTCAGCGA TGTTGCAAAC AGTGTCGTCG AACGGGCGCT GACCGAGTTC

1701 GGGCGGCTCG ACACACTGTT CAACGTCGTC GGCGGGAGCC TGGCCCGCAA

1751 CGTGGAGGAG ATCAGCGAGG AGCAGTGGCG CAGCCAGATC GACATGAACC

1801 TCGGCAGTGT CTTTCAGATG TCCAAACCCG TCATCCCACT CCTGCGCCAG

1851 GGTGGCGGCG GGTCGATCGT CAACGTCGCG TCGACGGCCG GGATCCTCGC

1901 CGAGAACAGG TGCTCCGCCT ACAGCGCAAG CAAAGGCGGG GTCGTGCTGC

1951 TCACGAAGAA CATGGCCCTC GACTTCGCCC GCGACAACAT CCGCGTGAAC

2001 GCGATCGCCC CCGGGGGCAC CCGCACGCCG AGGATCGAAC GGTTCCTGGC

2051 CGAGCACCCC GAACACGCCT CGATGATGGT GGACCTCTGC GCGATGCAGC

2101 GTTTCGCAGG ACCAGACGAG ATCGCGGCGC CGGCTGTGTT TCTTGCCTCG

2151 CCCGAGGCGT CCTACATCAC CGGTGCCGTA CTGCCGGTCG ACGGAGGCAT

2201 GACCGCCGGC GTCACTTTCC GGGCGTTCGA GGCGGTA**TGA** GCATGATCCC

2251 CAACCGCTGG AATCGGCACC GGACCCACGA AGCACCGCG AGCTGAG**ATG**

ADH-zinc

2301 GAAGCGATCG TTCTCAACGG CACCAACGAC GTCGGTCTGA CGTCGGTGCC

2351 AGACCCGGCG CCGCAGGACG GTGAGGTCAT CATCGAAGTG GCGGCGACAG

2401 GACTGTGTGG AACTGACCTC CACGAGTATG TCGCGGGGCC CACCTTCTCG

2451 CAGCCGCCGG TGGTGCTCGG TCACGAGGTC TCGGGCCGGA TCGTGGAGGT

2501 CGGAGCGGGC GTCGACCAAT CCCGCATCGG GGAGGGCGCC GCGGTGATCC

2551 CGATGGATTT CTGCGGGAGC TGCCACTACT GCCACCGGTC GCTCTACCAC

2601 CTGTGCCAGC GCCCAGGATG GATCGGCTTC ACCCGAAACG GAGGCCTCGC

2651 GAACTACGTC GCAGTGCCCT CTCGGCTCGC AGTCCGAGTG CCGGACGTGG

2701 TGGACCTCGA GGAGGCGGCG CTGACCGAGC CGACGGCGGT GGCGTTCCAC

2751 GCGGTGCGGC GAGCGGAACT GCTCCTCGGC GAAACGGTGA TGGTCCTCGG

2801 GGCCGGGGCA CTCGGGCTCA CCGTGATCCA GTGTGCACGC GCGGCCGGAG

2851 CTGCGCGAAT CTTCGTCACG GAACCAAGCG GCGTGCGGGC CAGCCTGGCG

2901 CGCGACCTCG GCGCCACGTT GGTGCTCGAT CCGCATGACC CCGGGACCAC

2951 CGCGTGCATC CTGGAGGAGA CCCGCGGCGT AGGGGTGGAC GTGGTCTTCC

3001 ATGTGGCGGG CAGCGCGGAG GCGTTCACAC AGGGCCTGGA CTGCCTCCGC

3051 AAACAGGGCC GTTTCATGGA GATGTCGTCG TGGGCCGGCG CGGCCTCGCT

3101 CGATGTCAAC CGCCATCTGC TCAAGGAGAT TCAGCTCCGG ATGGTTTTCG

3151 GTTACGACAT GTTCGACGAT TTCCCGGCCG TTCTCGCCCT GATCGCCGAC

3201 GGAAAACTCG CGCTCGCGCC GCAAATCACC GCTCGAGTCC CGCTGGACCG

3251 CGCCGTCAAG GAGGGATTGG GCGGGCTATT GGAGGGCCGG GAGGGTCTGG

3301 TCAAGGTGCT GGTGAAGCCG **TGA**GTGAGGG AGGCTTG**ATG** GTCGTCGCCG

orfN4

3351 CGACAAGCCG CGGTGTATTC ACCGTGTCCG GCGACGGCAA GGCCCGGGCC

3401 TGGCCCGAGT TGCCGGGGCA GGTCATGGCC ATGGCTGTCC AGGACGAGCG

3451 CGTCGCCGTC GCCGTCCACA AGCACGGCGT GTTTCTGGCC ACCGCGGGCG

3501 CAGACCACTG GACTGACCTC GGCGATGGCC TCGCCCACCG CGACGTGCGC

3551 GCGCTGGCGT TCGACCCGCA CACGCCCAAC GCGCTCTACG CCGGAACAGA

3601 ACCAGCGCAC TTGCTGCGGT GGCAAGACGG GATCTGGGCC GAGTGTGGAA

3651 ACGTCCTCGG CGTACCCGAA GCGAAAAGGT GGAGCTTCCC CATCCGCCCG

3701 GGAATCGCGC ACGTCCGTAC CATCGCCGTG CACCCACTAG AAGCTGACGT

3751 CGTCTACGTC GGCATTGAAG TTGGATCCCT CCTGATCACC CGGGACCGGG

3801 GACAGACGTG GGAGGAGATC GATGGCCTCG GTCACGACAT CCACCGCGTG

3851 GTCCTGCACC CGGAAGCTCC CGACCGGCTA ATCGTCACCA CCGGCCAAGA

3901 CAcCCAAGCC TTACCGCGGC GGCAAGGGCA TTTACCGCAG CACCGACCGC

3951 GGATCGAGCT GGGTGCAGTC CAACAACGGA CTTGGCGAGC GCAACTACAC

4001 CGAAGACGCG ATCGTGGTGC ATCCCGCCGA CCCCGACGTG GTGTTCCTCG

4051 CCGCGGCCGA CGGCATCCCA CCGAAGTGGG CCGCAGTGCG CCGGCTGGTG

4101 ATGGGCGTGC TCAACGGCAA CGTCTATTTC TTGTCGCCGT CCAAACTGCG

4151 CCGACGCCGC GGCGCCGACG TGGGATTCTT CCGCAGCAAC GACGGTGGCG

4201 CCTCCTGGGT ACGGCTGGAT ACCGACGACG ACCGCGGCCT GTTCGACATG

4251 GTCTGGGCGC TGGAAGGCGA GCTCAC**TGA**A CACCGTGGGT TTCGGCTGTA

4301 CTACGGCACC ACGGCGGGTG GGCTTTATGT GTCGGACGAC GAGGGCAACA

4351 CCTGGAAGTG CCTGGCCGAC GGCCTGGGCG CGGTCACCCA CATCGCGACG

4401 CCGAAGAAGG GAACAGCGCA **GTG**AAGCGTG GACCGACGCT GGAGTTCGAC

nidX

4451 CACATACAGC CGGCGATCCG AAAGCGGTTT ACGTCGGCCG CGGACATCCC

4501 CAAGGAGGTG TTTAGCGACC CGGACGTCTA CCGGGAAGAG CTCACTCGGA

4551 TCTTCTATGG TCCCTATTGG CATCCGATCG CGCATCGGGC CGAGCTGGCC

4601 GAGCGCAATG CCTTTCGGAC GAGATGGTTG GCTGATGTGC CGCTGCTGAT

4651 GGTGCGGGAC GGCGATGACC GCATCCGCGT GTTCGTCAAT TCCTGCGCCC

```
4701  ATCGGGGAAC GCTTCTGGAA CAGCGCCGGT GCGGGGTGGC GGAGCGATTC
4751  GAGTGTCCGT ATCACCGGTG GCTCTTCAAC AATGACGGCC GGTTCGCCGG
4801  CGCGCCCCGC CGCATGCAGT TTCGCCCGGA CTTTCGCGAG GAGGACTACG
4851  GCCTCCGGGA GCTGCACGCA GTCGAGGCGT GGGGTTTGAT CTTCGTCAGC
4901  ATGGCTGAGC AGCCGCCGCC GTTCGACGAT TATCTCGGCG ATAGCGCGGA
4951  TCCGTTGCGC GACTGCATGG TCGATGACGG GAACTTGACG TTGCTGGGCT
5001  ACCAGACGGT GGTGTTTCAG AGTAATTGGA AAACCTACAT CGACAACGAT
5051  CCCTATCACG CGCCGTTGCT GCACAGCGCG TTCAAACTGC TCAACTGGCA
5101  GGGCGGCAGC GGCAACGTCT TGGTCAGCGA GCCCTATGGG CACATGTCGA
5151  TTCTGTACGA TTCGCAGCCC TACGTGGACA ACGGTTTCCT GGCTGACCCG
5201  AGTGTGGTCA CGCGGATGGG GGATGACAGC CGGGCCCGCG TGATCGCGTT
5251  ACGGCCGGTT ACCGGGATCG TGCGTCACGT CGACACGATC AACGTACGTT
5301  ACGCTCGCCC GCTGGGGGTT GATCGCACCG AAGTGCGCTA CACGTTCTTC
5351  GGTCATGCCA GTGACACCGA GGACTTTGCG CGCCACCGCG TGCGCCAGTC
5401  GTCGAACCTG CTGGGGCCGA GCGGCTTCAT CAGTATCGAG GACGCCGCCG
5451  TCTACAACCG CGTGCAGGCG ACCGCGTGTG ATGGCGGCTA TCAGCGCTTT
5501  GTCGCCGGCG TCGGCCGACC GCTCTCGGAA TCGTCACAGA ACGACGAGGC
5551  CGCCAACACC GGATGGTGGG CCCACTACCG GGAGGTGATG GAGTTTTGTT
5601  GATATCG
```

* Start codons were highlighted in bold and underlined and stop codons were highlighted in bold.

Appendix 1-5. Complete nucleotide sequence of the *pdoA* locus (GenBank accession number: AF546905)

```
1     ACGAGGGCAC CAAGCCCGGC AAGAGCACCG GCGAGTCCGC GGAGCTGCGG
51    GATGCACGCC GTCGAATCAA GCTGTTGGAG CAGGAAAACG AGGTCCTGCG
101   CCGGGCGAGC GCGTATTTGT CGCAGGCCAA TCTGCCGGGA AAAGGTTCTA
151   C[1]CCGCTCGTG AACGAGCTCG CCGCCGACGG GGTTCCCGTC GCGGTGACGT
      orfP1
201   GCCGGGTACT CAAGCTCGCC CGCCAGCCCT ACTACCGCTG GCGGGCCAAC
251   TCGGTGACCG ACGCCGAGGT CACCGAGGCC TACCGAGCCA ACGCGTTGTT
301   CGACGCCCAT CGCGACGACC CGGAGTTCGG CTACCGCTAC CTGGTCGAGG
351   AAGCGTGCGC GGCCGGTGAA CCGATGGCCG AGCGCACCGG GTGGCGGATC
401   TGCTCAGAGC ATCGCCTATG GAGTGCGTTC GGTAAGAAAC GTGGCTAGAA
451   CGGCAAAGTG GGTCCGCCGG TGCATGACGA TCTCGTCGAG CGCGACTTCA
501   CTGCCGAAGG GCCAAATCAG TTGTGGCTTA GCGACATCAC CGAGCGTCCG
551   ACACGTGAAG GCAAGCTGTA CCTCTGCGCG ATCAAGGACG AATTCTCCAA
601   CCGCATCGTC GGGTACTGCA TGGACTCGCG AATGAAGTCG CGGTTGGCGA
651   CTACCGCGCT GAGCAATGCG GTAGCACGCC GCGGCGAGGT GGCTGGCTGC
701   ATTCTGCACA CCGATCGTGG ATCTCAGTTC AGGTCAAGGA GTTTCGTTCA
751   TGCTTTGGGT CGCTACGAGA TGGTCGGATC GATGGGCCGT GTTGGTGCGG
801   CCGGCGACAA CGCGGCCATG GAGAGCTTCT TCAGCCTGCT GCAGAAGAAC
851   GTACTGGACC GCCGCCGGTG GGACACCCGA GAGGAGTTGA GGATCGCCAT
901   CGTCACCTGG ATCGAACGGA CCTACCACCG CCGCCGACGC CAGACCGCCC
951   TCGGGCGGTT GACCCCGATC GAATTCGAAG CAATCATGAC CACACCGGCC
1001  AGTCAGGCCG CGTGACCGAA ACTGTCACCT GATCGTGCAG CAGACCCGTT
1051  CTTTGAGAGT CCAGGCCCGT CCGATGCGTC GGTTGGTACG GGTGATCTGG
1101  TTGACCAATG CGTGTTTGTC ATCGGTGGGT TTGTTCTCCC GGTGCGCAAT
1151  GCTCAGCGTG TGGTCCACCT TTGCCCAGCG CTCATCGCCA CTTTGTCCGG
1201  GCCTGCGGCG GCTTCGGCGA ATACCGGTCG AGGGCGCGGT TGACCTATCG
1251  CGTGATGTGG AAGTCGTCGT AGCAGATGAC CGCGTGCGGG ACTGCTGCGG
1301  TCGTGACACC GGTGCAGGCG CGCGAGACGT CAATGCTGAC GGTTTCGATA
1351  GCGGCCAGGG CTGAATCTGG CTGGCTGGCA CGGAAGTTGG CAAGCGATTC
1401  TTCGCTGCGT CCGGGTTGGA TGTCGACCAC TGCGCCGGTG TCGTGGTCAC
```

1451 CGATGATGGT CAGATACCGA TGCGGGTGGC GATAGCAGAT CTCATCGACA

1501 CCGATGCGGT AGAGCGTCTG CAAGAGGCGT TGGTCCAACA GTTAGGCCCC

1551 ACCGCGGTTG ATGATCGCAG AGACGGTCTC CCAGGCGTAG CCCATCAACG

1601 TGGCCACGAG GTTCGGTCGG TGCGCTGAGC TAGCCACACC GCATGTCCTC

1651 GAAGTCACGG GTATGGCGGG CGCTGGGAAG AGCCGAAGGC AGCTCCTCGG

1701 TGACGATCCC GCAGTCCGAA CAGTCCAGCT GGCGGATTTC GTAGACCAGC

1751 CACAGCCGCG CCCTGGCCAG ATCGAGATGG CGCCAGCGCC GGCGGCGTCA

1801 GGTCATCGAC CGCCGGTAGC CGTTTGCCGC ATGGGCGACA CAACAGGTGG

1851 GCCTTCGGGC GCAGCCGGAC CTCAATATGA CCGTCATCAA TGACGACGTC

1901 GGTAACTGAA CCGCCTGTAA GTCTGTACAT GGCCGGCCCG GTGCTGGCTG

1951 ACAGGTCGGT GCCGCATATG AAGACTTACA GGCGGTTCAG CTGCTCACGA

2001 GCCTCAGGAT CAATAACAGT CATCAGTGTC GACTCTCCGG CAGGACACCC

2051 CACCCCCCTA CACAAACCAT CTGACACCTC CGCGTGCGCG TCCCTCATTT

2101 CGCCGCCGAA CCATCAATTC CTCATGCAGC ACAACACCAA CAGTCGCTGA

2151 CCAGGACCAA AGCGCACCTA GAACCCTAGG AGCGCCACAT GGGTTCGTGC

2201 TGGCTCTGTA CGACGACCCT TATTTCATCG GGCGGGAGAC CGGCCCCTTT

2251 GACCAAGTTC CCGAAGCGAC TGCACCGCGT CCAGGGACTT GACCGAGATC

2301 TCTCTTGACG CGCAAGTGAG ATCTGGCTAA CATTCTCTAC AGCGGAGGAC

putative promoter site

2351 TTATCTGTTC AGCAGAACAG GCTGGTCAGT ATTGACGGTT TGCCGGAGGA

SD

2401 GTTCGATTTG ATGAACGCGG TCGCGGTGGA CCGGGATACC CGTGAGTCCG

pdoB

2451 TCGAGGACTT CATGTTTCGG GAAGCGGAGT TGCTCGATGG AGGACAGTTC

2501 CGGGACTGGT TGGGCCTGCT TGACCCCGAC ATCCGGTATG TGGTTCCGGT

2551 GCGCACAACC CGGGAGGACT CCGCGGGTTG GGTGAGCGCG ATCGCGCACT

2601 GGAACGACGA TTACACGGGC TTGGAGATGC GGGTCCTGCG CAGCGAGACG

2651 GAGTTCTCAT GGGCGGAGTC ACCTCGATCC CGGACCCGCC ACTTCGTGTC

2701 CAATATCCGC ACCACTGCCG GACCTGGGGC TGATGAGTTG ACCGTGCGGT

2751 CGAACCTGTT GTTCTTCCGC AGCCGCGGAG CTAGCGGACG GTGGGAGTTG

2801 CTGTCGGCTG AACGCGTCGA CGTGCTGCGC CGGAGCGACG GGTCGCTGCG

2851 GCTCGCTCGG CGTGAGGTCT TGCTCGACCA TTCGACGCTG CCTATCGACA

2901 ACCTGTCGGT AGTCCTGTAA GCCACGCTCT CTCGCCGTCG TCGTCCTTGA

2951 AAGCACTGCC TGTCCAGAAA GGGTCCGATC AC**ATG**ACCAC CGAAACAACC

pdoA

3001 GAGACCACCG GAACCGCTGG CACGAGTGAT CGCTACCTGC GGCGCGCGTT

3051 GCGCGACGTA GCGGACGGGC TCAAGGTCGG GCGACTACCC GCACGCGTCG

3101 TCAGCGATCC GACGCTCCAT ACGATTGAGA TGGAGCGGAT CTTCGGGCGC

3151 GCCTGGGTGT TCCTCGCGCA CGAGTCGGAG TTGGCCAAGT CCGGCGACTT

3201 TGTGGTGCGT TATATCGGGG CCGATTCGGT GATCGTCTGC CGGGACGCCT

3251 CCGGCCGCAT CCAGGCGCTG TCCAATTCTT GTCGCCACCG TGGCGCTCTC

3301 GTGTGCCGTG CGGAGACGGG AAACACCGCC CACTTCCAGT GCCCGTACCA

3351 CGGCTGGGTG TACAGCAATA CCGGGGATCT CGTCGGTGTG CCGGCGATGA

3401 GTGAGGCCTA TCCCGGCGGT TTCGATAAGA CGCAGTGGGG ATTGCGCCAC

3451 ATCCCCCATG TCGACTCGTA TGCCGGATTC ATCTTCGGTA GCGTCGATCC

3501 GAAGGCGCCG AGCCTGACCG ACTATCTCGG CGACACGACG TTTTACCTCG

3551 ACCTCATTGC GAAGAAGACA GCGGGCGGTC TGGAGGTGAT AGGGGCGCCG

3601 CATCGGTGGA CTATGTCTGC GAACTGGAAG ACGGCCGCCG ACAATTTTGT

3651 CGGCGACTCC TACCACACCC TTTTCGCTCA CCGCTCTATG GTGGAGCTGG

3701 GCATGGCTCC GGGTGACCCG AACTTCGCGA GCGCACCCGC GGAGATCTCG

3751 CTGCAGAACG GTCACGGCGT AGGTGTGCTC GGGTTTCCGC CCACGCTCGC

3801 CGATTTCCCA GAATACGAGG GATACCCCGA CGAAGTCGTC GACCAGATGG

3851 CGGCGTCCTA CCCGTCTTTG GCGCACAAGG ACATGATGCG ACGCTCAGCC

3901 TTCATTCACG GCACGGTGTT CCCAAATTTG TCCTTCATCA ATGTGACCAT

3951 CGCGCCTGAC CACATGTCGC CCCCCACCCC GTTTATCACG TTCCGGTTGT

4001 GGCAGCCGCT ATCTCATGAC CGGATGGAGG TCCTTTCCTG GTTCCTGGTC

4051 GAACGCGATG CTCCGGAGTG GCTACGCGAG GCGTCGCAGG CCTCCTATGT

4101 CAACAACTTC GGTCCGGCCG GTGTTTTCGA ACAGGACGAC GCGGAGGCAT

4151 GGAAGGCCAT CACGGAATCT GTCCAGGGTC CGTTCGCCGG TGAAGGACTC

4201 CTGAACTACG AGATGGGCAT GGACCTGACT CCACTTAATG ACTGGCCAGG

4251 ACCGGGCGAA GCCCTCCCGA GTGGGTACGC CGAGCAGAAC CAGCGACGGT

4301 TCTGGGGGCG ATGGCTGGAC TACATGAGCC AACCTGCCCA GTTCCGCGGC

4351 GGTGCTCGA**T** **GA**CGATTCTG CCCAGGCTGC TGTCACGATG TACGCCGGTC

4401 CAACGATCGA CCGCAGGTGA GTGATGCGGA GGCGGGAGAC CGCCGAGTGG

4451 CAATAGTCAC TGCCGCCGCG GCAGGGATCG GTAGAGCCAT CGCCGCTCGG

4501 TGGTGTCGTG ACGGCGGGAG CTGTGTC**ATG** GCCGACACCG ACGCCGGGCG

ADH-short

4551 CCTCGACGCA GCAGTTGCCG AACTGGCCGA GTCCGGCGCC GCGGTATGCG

4601 GCGTCGTAGG CGACGTGTGC CGCCGTGATG TCGCTGACGG CGTCGCCGAA

4651 CGGGCGCTGA CCGAGTTCGG GCAGCTCGAC GCCCTTTTCA ACGTCGTCGG

4701 CGGGAGCCTG GCCCGCAACG TCGAGGAGAT CAGCGAGGAG CAGTGGCGCA

4751 CTCAGCTCGA CATGAACCTC GGCAGCGTCT TTCAGATGTC CAAACCCGCC

4801 ATTCCCCTGC TTCGCCAGGG TGGCGGCGGA TCGATTGTCA ACGTTGCGTC

4851 GACGGCGGGG TTACTAGCCG AGAACAGGTG CTCCGCGTAC AGCGCGAGCA

4901 AAGGTGGGGT CGTGCTACTC ACGAAGAACA TGGCCCTCGA CTTCGCTCGT

4951 GACAACATCC GCGTCAACGC AATCGCTCCC GGAGGGACCC GCACGCCGAG

5001 GATCGAACGG TTCCTTACCG AGCACCCCGA ACACTCCTCG ATGATGGTCG

5051 ATCTTTGCGC GATGCAGCGT TTCGGTTGGC CGGACGAGAT CGCTGCGCCG

5101 GCTGTGTTTC TTGCCTCGCC CGAGGCGTCT TACATCACCG GTGCCGTCCT

5151 GCCGGTCGAC GGAGGCATGA CCGCCGGCGT GACTTTCCGG GCGTTCGAGG

5201 CGGGA**TGA**GC ATGATTCCCA AGCGCTGGCA TCTTTGCCGG ACTCACGACG

5251 AGGCCGGGAG CTGAG**ATG**GA CGCGATCGTA CTCAAAGGCA CCAACGACGT

ADH-zinc

5301 CGGCCTGACA TCGGTCCCGG ACCCCGCGCC GCAGCCTGGT GAGGTGATTA

5351 TCGAAGTGGC GGCGACAGGG CTGTGTGGGA CCGACCTGCA CGAGTATGTG

5401 GCGGGACCCA CCTTTTCGCA GCCACCGGTA GTGCTCGGCC ACGAGGTATC

5451 GGGCCGGATC GTGGAGGTCG GTGCGGGCAT CGACCGATGC CGTATCGGGG

5501 AGGGCGCCGC GGTGATTCCA ATGGACTTCT GCGGCAGCTG CCACTACTGC

5551 CACCGGTCGC TCTACCACTT GTGTCAGCGC CCAGGATGGA TCGGCTTGAC

5601 CCGGAACGGC GGCCTGGCTA GCCACGTCGC AGTGCCGTCG CGCCTCGCGG

5651 TCCGAGTGCC CGACGCGGTG GACCTCCAGG AGGCGGCCCT GACCGAGCCG

5701 ACGGCAGTGG CGTTCCACGC GGTGCGGCGA GCGGAGCTGC TCCTCGGCGA

5751 AACGGTGCTG GTCCTCGGCG CCGGGGCGCT CGGGCTCACC GTGATCCAGT

5851 GCGCACGCGC AGCAGGAGCC GCGCGGATCT ACGTCACGGA ACCGAGCCGC

5851 GTGCGCGGCA ACTTGGCCCG CGACCTCGGC GCCACGTTGG TGCTCGATCC

5901 ACACGAGCCC GGGACCACCG CGCGAATTCT GGAGGAGACA CGAGGCGTTG

5951 GGGTGGATGT CGTGTTCCAC GTGGCAGGCA GCGTGGAGGC GTTCACACAG

6001 GGCCTGGACT GCCTACGCAA ACAGGGCCGC TTCATGGAGA TGTCGTCATG

6051 GGCCGGGCCG GCCTCGCTCG ATGTCAACCG CCATCTTCTC AAAGAGACGC

6101 AGCTCCGGAT GGTTTTCGGG TACGACATGT TCGACGACTT CCCCGCCGTC

6151 CTAGCGCTGA TGGCCGACGG AAAAATCGCG CTCACGCCGC AGATCACCAC

6201 CCGAATCCCA CTGGACAGTG CGGTGGAGGA GGGGTTGAAC GGGCTGTTGG

6251 AGGCGCGAGA GGGTCTGATC AAAGTGCTGG TGAGGCCA**TG** **A**GTGGGGTTG

6301 TAGGTCGAGT GATGGCGGTG TCGATGCCGG AGTGCGACTC TTTGGCGGTT

6351 GGCGGGCCCA CAATCTTGGT GCCGTGTTGG TGATCTGCGA CCCCGTCCGT

6401 TCCAAAGTGG AGCACCCTGG CGAGGGCGGC GGCTCGAGTT CAGATGAGGA

6451 GGGGTCCTG**A TG**CCCGTCGT AGCGGCAAGT CGGGGCGTAT TCACTGTGTC

orfP6

6501 CGGTGAGGGG AAGGTCCAGG CCTGGCCCGC TTTGCCGGGG CAGGTAATGG

6551 CCATGGCCGC GCGCGGCGAT CGCGTCGCCG TCGCCGTCCA CAACCAGGGC

6601 GTATTTCTGG GTACCGCGGG TGCTAACTGC TGGACTGGGC TTGACGATGG

6651 GCTCGTACAC CGCGATGTGC GCGCGCTGGC GTTCGACCCG CACGCACCCC

6701 ACGCGCTCTA CGCCGGCACA GAACCAGCGC ACCTGCTGCG GTGGGAAGAC

6751 GGGACCTGGG CCGAATGCGG GAACCTGCTC GGCGTGCCCG AAGCGAGAAG

6801 GTGGAGCTTC CCCATCCGCC CCGGAATCGC GCACGTGCGC ACCATCGCCG

6851 TGCACCCATT GGAAGCTGAC GTTGTCTACG CCGGCATTGA AGTCGGATCC

6901 CTTCTAGTCA CCGGGGACCG GGGACAGACA TGGGAGGAGG TCACCGGCCT

6951 CGGTCACGAC ATCCACCGCG TGGTCCTGCA CCCCGGGGCT CCCGACCGGA

7001 TCATCGTCAC AACAGGCCAG GACACCAAGC CATACCGCGG CGGCAAGGGC

7051 CTGTACCGCA GCACCGACCG CGGATCGAGC TGGATGCAGT CAAATGACGG

7101 ACTCGGAGAG CGCAACTACA CCGAAGACGC GATCGTGGTC CACCCAGCCA

7151 AACCCGAGGT GGTATTCCTC GCCGCAGCCG ACGGCATCCC ACCAAAGTGG

7201 GCCGCAGTGC GCCGCCTGGT AATGGGCGTG CTCAACGGCA ACGTCTATTT

7251 CCTGTCGCCG TCCAAGCTGC GCCGACGCCG CGGCGCCGAC GTGGCGTTCT

7301 TTCGCAGCGA CGACGCTGGC GCTTCCTGGG GTACGGCTGA ATACCAACGG

7351 CGAGCCTGGC CTGTTCGACA TGGTCTGGGC GCTGGAAGGC GAGCTCAC**TG**

7401 **A**ACACCGTGG GTTTCGGCTG TACTACGGCA CCACGGCGGG TTCGCTTCAC

7451 GTGTCGGACG ACGAGGGCAA CACCTGGACG CGCCTCGCTG ACGGCCTGGG

7501 CGCGGTCACC CACATTGCGG CCCTACAGAA AGGAATGGAG TT**GTG**AACGG

pdoX

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7601 TTACGTCGGC CGCGGACATC CCCAAGGAGG TGTTTAGCGA CCCGGACGTC

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7851  ATGCGGGGTG GCGGAGCGAT TCGAGTGCCC GTATCACCGG TGGCTCTTCA
7901  CCAACGACGG CCGCTTCGCT GGGGCGCCTC GCCGCATGCA GTTCCGGCCG
7951  GACTTCCGCG AGCAAGACTA TGGCCTACGG GAGTTACACG TCGTCGAGGC
8001  GTGGGGATTG ATCTTTGTCA GCATGGCCGA CCAGCCGCCA CCGTTCGCCG
8051  ATTTCCTCGG CGATAGCGCC GGACCGCTGC GCGACTGCAT GGTCGATGAC
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8201  CGTTCAAACT GCTCAACTGG CAGGGCGGCA ACGGCAACAT CTTGGTCAGC
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8451  CGAAGTGCGC TACACGTTCT TCGGTCATGC CAGCGACAGC GAGGACTACG
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8551  ATCAGCATCG AGGACGCCGC CGTCTACAAC CGCGTGCAGG CGACCGCGTG
8601  TGATGGCGGC TACCAGCGGT TCGTCGCCGG CGTCGGCCGA CCGCTCTCGG
8651  AATCGTCACA GAACGACGAG GCCGCCAACA CCGGATGGTG GGCCCACTAC
8701  CGGGAGGTGA TGGAGTTTTG TT
```

* Start codons were highlighted in bold and underlined and stop codons were highlighted in bold.

[1] a putative start codon for *orfP1*.

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