Comparing Cultivation-Dependent And Cultivation-Independent Approaches For Characterizing Activated Sludge And Soil Bacterial Populations Degrading A Model Pollutant, 2-Chloropropionic Acid (2MCPA)

A thesis presented by

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Declaration

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Abstract

Extensive use of organic halogenated chemicals in many areas of agriculture and industry has resulted in widespread presence of toxic and carcinogenic compounds in the environment, creating many problems. Bacteria are able to degrade these harmful compounds, primarily by production of dehalogenating enzymes using them as sole sources of carbon and energy. Little is known about microbial adaptation during α-haloacid (αHA) degradation. Therefore, this study aimed to investigate bacterial community and dehalogenase genes during degradation of 2-chloropropionic acid (2MCPA) in activated sludge and soil inoculated Ready Biodegradation Tests (RBT) format, using DNA based cultivation-independent and cultivation-dependent methods.

Changes in the bacterial communities of activated sludge (AS) and soil (HS and LS) RBTs were monitored by analysis of bacterial 16S rRNA and αHA dehalogenase (*dehI* & *dehII*) genes. Bacterial 16S rRNA gene DGGE profiles showed that several bacterial species (phylotypes) were persistent during 2MCPA degradation, whilst other phylotypes were either enriched or appeared transiently.

Some changes in the bacterial communites were associated with appearance of *deh* genes. At lower inoculum concentration of soil (LS-RBT), 2MCPA degradation gave biphasic curve of dechlorination; the presence of a *Comamonas* phylotype during the initial stages of substrate degradation coincided with the presence of group II dehalogenases (*dehII*), and was replaced by *Methylibium* in the second phase of 2MCPA degradation, coinciding with the presence of a group I dehalogenases (*dehI*). However, in the high inoculum of soil (HS)-RBT and AS-RBT, 2MCPA degradation was completed more rapidly in a single phase, with enrichment of *Herbaspirillum*-like (in HS-RBT) phylotypes, and *Caulobacter* and *Cupriavidus*-like (in AS-RBT) phylotypes.

Samples removed periodically from the RBTs were used to inoculate solid and liquid sub-cultures (2 or 10 mM 2MCPA) for further enrichment and isolation of 2MCPA-degrading bacteria. DGGE profiles of 16S rRNA gene sequences from the AS-RBT revealed considerable differences from those of the liquid sub-cultures, suggesting that degradation of 2MCPA was affected by and possibly determined by substrate concentration and presence of specific degraders in a system.

The isolates obtained from soil and AS-RBTs, whether or not they reflected the RBT, were mainly *Alphaproteobacteria*, indicating the importance of these bacteria in degrading 2MCPA. Four 2MCPA-degrading bacteria, *Afipia* sp. KH3, *Rhizobium* sp. KH31, *Herbaspirillum* sp. KH17 and *Methylobacterium* sp. KH4, were isolated in this study and characterized phylogenetically and with respect to their dehalogenases. Among the genes involved in 2MCPA degradation, the sequence of *dehI* of strain KH17 was identical to that of *dehI* of *Herbaspirillum* sp. DA1.

This study showed there is significant bias during liquid sub-culture process, and highlights the advantage of using molecular based (PCR-DGGE) methods in investigating the actual contribution of bacteria population in degrading xenobiotics (αHAs). Moreover, molecular genetic analyses were useful in monitoring the enrichment and isolation possesses.

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Abbreviations

2MCPA – 2-Monochloropropionic acid

2MCBA – 2-Monochlorobutric acid

22DCPA – 2,2-Dichloropropionic acid (Dalapon)

2,4-D – 2,4-Dichlorophenoxyacetic acid

αHA – α-halocarboxylic acid

γ-HCH – γ-hexachlorocyclohexane (lindane)

BLAST – Blast local alignment search tool

CHQ – Chlorohydroquinone

DCA – Dichloroacetic acid

DGGE – Denaturing gradient gel electrophoresis

dNTPs – Deoxyribonucleotide triphosphates

DTT – Dithiothreitol

EDTA – Ethylenediaminetetraacetic acid

HAD – Haloacid dehalogenase

HGT – Horizontal gene transfer

IPTG – Isopropyl-β-D-thiogalactopyranoside

IS – Insertion sequence

MCA – Monochloroacetic acid

OECD – Organization for economic co-operation and development

PAGE – Polyacrylamide gel electrophoresis

PCR – Polymerase chain reaction

RAPD – Random amplified polymorphic DNA

RBT – Ready biodegradation test

RDP – Ribosome database project

SBS – Standard basal salts

TAE – Tris acetate acid EDTA buffer

TCA – Trichloroacetic acid

TEMED – Trtramethyleethylenediamine

WWTP – Waste water treatment plant

X-Gal – 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

1. General Introduction

1.1 Halogenated compounds in the natural environment

Halogenated compounds are compounds that contain carbon-halogen bond (including chlorine, fluorine, bromine, and iodine atoms). Large scale synthesis and extensive use of organic chemicals in many areas of agriculture and industry have led to widespread distribution of harmful compounds in the environment and created extreme pollution problems. These chemicals, which are produced industrially and introduced into an environment as novel compounds, or at concentration that exceeds that normally present, are called xenobiotics (Top and Springael, 2003). Many are toxic to biota, pose a threat to human health and are considered environmental pollutants. Halogenated compounds, which constitute >75% of compounds listed as "priority pollutants" by United Kingdom Environment Agency (Ground water Directive, 2006/11/EC: www.wfduk.org./jaqdaq/), have been used widely and released to the environment by industry, and agriculture as herbicides, pesticides, fungicides, solvents, plasticizers, paints, printing ink, adhesives, hydraulic and heat transfer fluids, flame retardants, refrigerants, additives for cutting oils, textile auxiliaries, and intermediates for chemical synthesis (Fetzner and Lingens, 1994; Janssen et al., 1994b).

In addition to industrially synthetic organohalogen compounds, many halogenated organic compounds are present naturally in the environment (Gribble, 1996; Laturnus et al., 2005; Teuten and Reddy, 2007). More than 3800 organohalogens compounds are produced biologically or by natural processes such as eruption of volcanoes, forest fires, and other geothermal processes (Gribble, 2003). In addition, marine organism such as seaweeds, sponges, corals, tunicates and bacteria are major organohalogen producers. Also, terrestrial plants, fungi, lichen, bacteria, insects, some higher animals, and even humans produce organohalogen compounds (Gribble, 1994, 1998, 2003).

Organic chemicals that persist in natural environments or are degraded very slowly, owing to the inability of microorganism to degrade them, are known as recalcitrant compounds (Alexander, 1981). These chemically synthesized organic compounds are not readily degraded in the environment, and many accumulate in soil water,

groundwater, lake and river water (Esteve-Nunez et al., 2001; Pervova et al., 2002). Besides the local and regional contamination of soil and ground water by these compounds, halogenated contamination continues to be a global issue, partly because transport through water and air helps the compounds to spread across the Earth (Iwata et al., 1993; Connell et al., 1999).

The persistence of these compounds in the environment causes considerable environmental pollution and human health problems because of their toxicity and bioaccumulation in food chain and ground water (Brokamp et al., 1997; Klier et al., 1999; Squillace et al., 1999; Stackelberg et al., 2004; Ojo, 2007; Dórea, 2008). It is well known that some halogenated compounds form toxic intermediates which may affect cellular metabolic processes (Slater et al., 1995; Janssen et al., 2001). For example, monochloroacetic acid (MCA) which acts as herbicide by inactivating glyceraldehyde-3-phosphate dehydrogenase (Sakai et al., 2005), causes sever tissue damages and systemic poisoning (Kulling et al., 1992; Kato et al., 2006). Iodoacetic acid (MIA) is a protein inhibitor (Carne et al., 1976), and MCA and MIA and fluoro acetate (MFA) caused acute toxicity in mammalian tissues (Hayes et al., 1973). These compounds are also known to form intermediates that can be toxic to microorganisms that have potential to degrade them (Janssen et al., 1994b; Janssen et al., 2005).

The halogen constituent is usually responsible for the toxic and recalcitrant character of halogenated compounds (i.e. as halogenation of compounds increases, toxicity and recalcitrance to degradation increases); the type of halogen atom(s) carried and its position within the compound often make these compounds relatively stable (Fetzner and Lingens, 1994). Removal of the halogen from these compounds usually reduces resistance to biodegradation and the risk of forming toxic intermediates during subsequent metabolic steps (Janssen et al., 1994b; Janssen et al., 2001). The environmental persistence of chemicals can be determined by measuring their biodegradability (Struijs and Van Den Berg, 1995; Ahtiainen et al., 2003; Rudén and Hansson, 2010).

Slow degradation of halogenated compounds in nature may be caused by a number of factors, including: lack of suitable catabolic enzymes (Pries et al., 1994a); toxicity of bio-available organic pollutants to the microbial populations; compounds are not available to microorganisms because of their hydrophobicity and persistence in water

or soil (i.e. concentration of the substrate in environment affect microbial attack to the substrate as low concentration or solubility of a organic chemicals may affect persistence (Boethling and Alexander, 1979); lack of transport of a molecule across the microbial cell membrane; presence of alternative nutrients; complexity of a substrate i.e. a readily degradable compound combined with resistant chemicals (Alexander, 1981; Paul et al., 2005). Moreover, the slow degradation of halogenated compounds may be due to unfavorable physiochemical parameters such as pH, temperature, and oxygen (O_2) (Laturnus et al., 2005).

1.2 Involvement of dehalogenases in the microbial degradation of halogenated compounds

Dehalogenation is the process of cleaving carbon-halogen bonds and the release of halide ions. Dehalogenation can be achieved abiotically, for example by photolysis (Lifongo et al., 2004), or in anaerobic and aerobic catabolic reactions catalysed by microbial enzymes generally called dehalogenases (Janssen et al., 1994a; Janssen et al., 2001; Van Pée and Unversucht, 2003; Grostern and Edwards, 2009).

Microorganisms are known to be able to degrade halogenated compounds in nature and play a major role in biodegradation and decontamination of environments contaminated with halogenated compounds (Weightman and Slater, 1980; Alexander, 1981; Chaudhry and Chapalamadugu, 1991; Fetzner and Lingens, 1994; Janssen et al., 1994b; De Lorenzo, 2008). Microbes degrade halogenated compounds in order either to exploit them for growth as a carbon source and/or as a means of protection against the toxicity of these compounds (Muller and Lingens, 1986). There are several key requirements for a microorganism to degrade xenobiotics: (a) the ability to transport the compound in to the cell where enzyme action can occur; (b) degradative catabolic genes must be expressed producing functional enzymes; (c) and the product of enzyme must be able to enter metabolism (i.e. to be a growth substrate) (Weightman and Slater, 1980; Weightman et al., 1985). Dehalogenases are key enzymes in the degradation of halogenated compounds (Fetzner and Lingens, 1994; Janssen et al., 1994b).

The term, dehalogenase was first used by Jensen to describe dehalogenating enzymes produced by bacteria and fungi that were able to grow on chlorinated organic acids

(Jensen, 1957; Jensen, 1959). Since then many different types of dehalogenases involved in the degradation of halogenated compounds have been found, purified, and characterized. They are classified into oxygenolytic, reductive and hydrolytic dehalogenases (Figure 1.1) (Goldman et al., 1968; Fetzner and Lingens, 1994; Janssen et al., 1994b; Fetzner, 1998; Janssen et al., 2001).

1.2.1 Oxygenolytic dehalogenases

Oxygenolytic dehalogenation reactions are catalyzed by monooxygenases (or dioxygenases) which incorporate one (or two) atoms of molecular oxygen to the substrate. For example, oxygenolytic dehalogenation of 1,10-dichlorodecane degradation (1,10-DCD) is catalyzed by a monooxygenase of *Pseudomonas* sp. 273 (Wischnak et al., 1998; Heath et al., 2006); trichloroethelene dechlorination is catalyzed by 2,3-dioxygenase of *Pseudomonas putida* F1 (Wackett and Gibson, 1988); dechlorination of chloroaromatic compounds is catalyzed by 4-chlorocatechol 1,2 dioxygynase of *Rhodococcus opacus* 1CP (Olaniran et al., 2001; Ferraroni et al., 2002; Ferraroni et al., 2004); and 4-fluorophenol dechlorination is catalyzed by 4-fluorophenol monooxygenase of *Arthrobacter* sp. strain IF1 (Ferreira et al., 2008). Cleavage of chlorohydroquinine (CHQ) by 1,2-dioxygenase LinE, forms maleylacetate during γ-hexachlorocyclohexane (γ-HCH) degradation (Miyauchi et al., 1999; Nagata et al., 2007). Dehalogenation of 4-chlorophenylacetate to 3,4-dihydrophenylacetate by *Pseudomonas* sp. CBS3 occurs via direct oxidative attacks of the aromatic ring by a two component 3,4-dioxygenase system, which require NADH (Markus et al., 1984; Markus et al., 1986).

1.2.2 Reductive dehalogenases

Reductive dehalogenation has been reported under anaerobic (DeWeerd et al., 1991) conditions, where the halogen constituent is replaced by hydrogen (Fetzner and Lingens, 1994), and can be achieved through three main routes (Figure1.1) (El Fantroussi et al., 1998): (a) Dehydrodehalogenation; for example found in haloalkane degradation e.g. the reduction of tetrachloromethane by fumarate-respiring *Escherichia coli* K-12 (Criddle et al., 1990); (b) Glutathione S-transferase reductive dehalogenases, e.g. the glutathione-dependent reductive dehalogenase LinD of

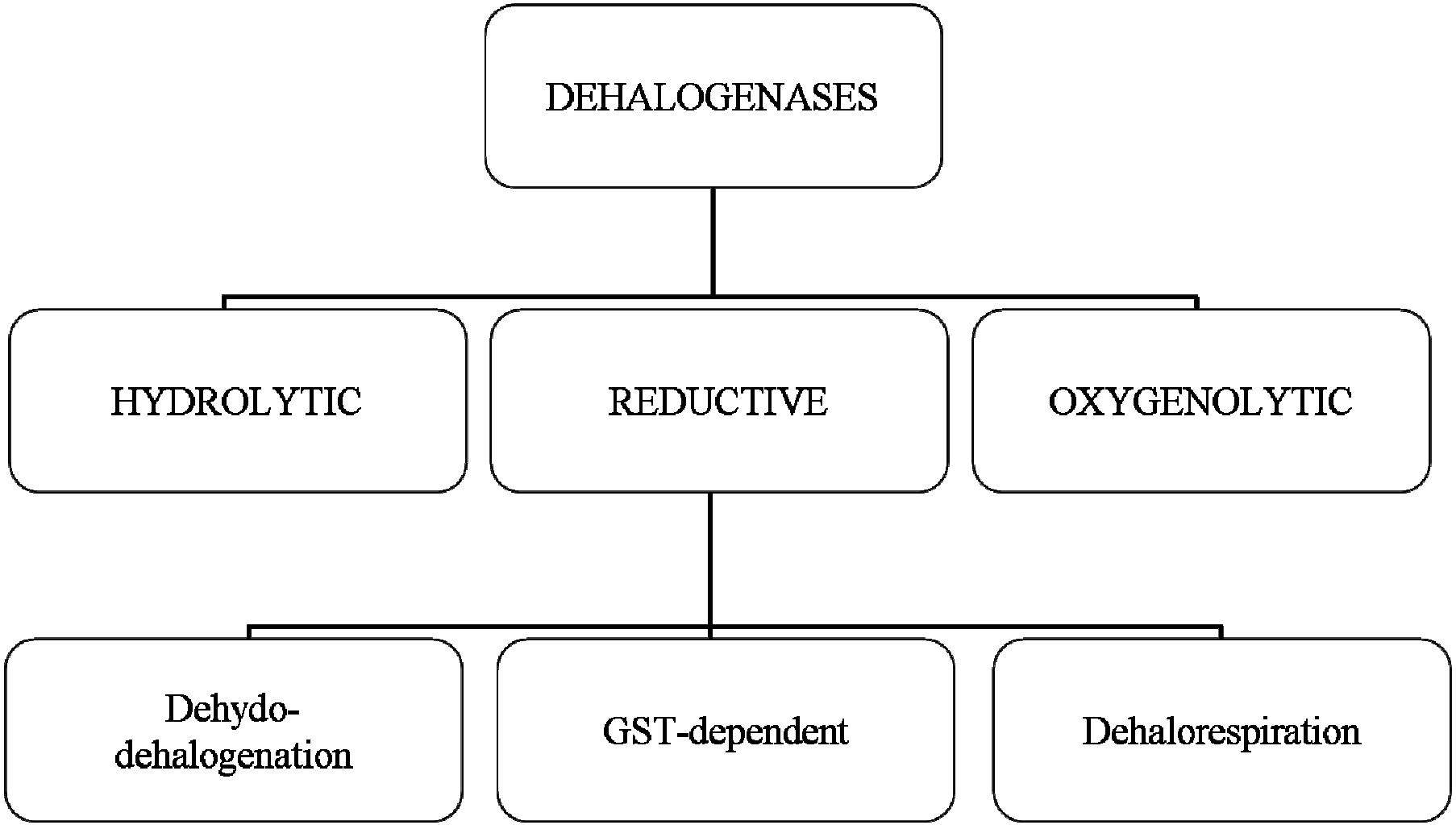

Figure 1.1 Types of dehalogenases

Sphingomonas japonicum UT26 catalyses the reductive dechlorination of 2,5-dichlorohydroquinone (2,5-DCHQ) to chlorohydroquinone CHQ, and CHQ to hydroquinone HQ during the degradation of γ-HCH (Miyauchi et al., 1998); (c) Dehalorespiration is a process in which bacteria use halogenated compounds as an electron acceptor during anaerobic respiration; e.g. the anaerobic sulphate-reducing bacterium *Desulfomonile tiedjei* strain DCB-1 utilizes formate or hydrogen as electron donor and 3-chlorobenzoate as electron acceptor, catalyzed by the membrane-bound 3-chlorobenzoate-reductive dehalogenase (Shelton and Tiedje, 1984; DeWeerd et al., 1990; Dolfing, 1990; Mohn and Tiedje, 1990, 1991; Ni et al., 1995; Holliger et al., 1998; Field and Sierra-Alvarez, 2008). Other examples of dehalorespiring bacteria are *Desulfobacterium dichloroeliminans* strain DCA1, which uses 1,2-dichloroethane as a terminal electron acceptor with formate or hydrogen as an electron donor (De Wildeman et al., 2003), and *Dehalococcoides* spp., which carry out complete anaerobic reductive dechlorination of tetrachloroethane and tetrachloroethene to the non toxic end product ethene (Futagami et al., 2008; Rossetti et al., 2008).

1.2.3 Hydrolytic dehalogenases

Hydrolytic dehalogenases catalyse the cleavage of carbon-halogen bond in which the halogen is replaced by hydrogen or hydroxyl group (Goldman, 1965; Goldman et al., 1968; Motosugi and Soda, 1983; Hardman, 1991; Van der Ploeg et al., 1991; Fetzner and Lingens, 1994; Slater et al., 1997; Kurihara et al., 2000). Hydrolytic dehalogenases are often associated with metabolism of haloaliphatic compounds; for example, haloalkane and haloacid dehalogenase. Several bacterial isolates able to dehalogenate chloroaliphatic compounds via hydrolytic dechlorination have been reported, and their enzymes have been characterized (Goldman, 1965; Kawasaki et al., 1981c; Kawasaki et al., 1981b; Motosugi et al., 1982a; Motosugi et al., 1982c, b; Allison et al., 1983; Ji-Sook et al., 2003; Kurihara et al., 2003; Omi et al., 2007). Haloalkane dehalogenases and haloacid dehalogenases have been isolated from both Gram-negative and Gram-positive bacteria (Olaniran et al., 2001; 2004). These are discussed further in sections 1.3 and 1.4.

1.3 Haloalkane dehalogenases

Haloalkane dehalogenases (HLDs) are microbial enzymes that catalyze the hydrolysis of haloalkanes to the corresponding alcohol, a halide ion and a proton as the reaction products (Fetzner and Lingens, 1994; Janssen et al., 1994b). According to structure and amino acid sequence similarities, the HLDs belong to the α/β-hydrolase fold protein superfamily (Ollis et al., 1992; Janssen et al., 1994b), and cleavage of the carbon-halogen bond proceeds via formation of covalent alkylenzyme intermediate (Janssen et al., 1988; Hardman, 1991; Newman et al., 1999; Janssen, 2004; Kurihara and Esaki, 2008).

The three-dimentional structures of the three HLDs (DhlA, DhaA, and LinB) have been reported, revealing two common domains: the α/β-hydrolase core domain (domain I; which showed highly conserved region and structure similarity with other hydrolytic proteins of α/β hydrolase superfamily), including the catalytic site; and the cap domain (domain II), which is proposed to play a role in determining substrate specificity that was significantly diverse among different dehalogenase (Pries et al., 1994b; Damborsky and Koca, 1999; Prokop et al., 2003).

In general, aerobic dehalogenation of haloalkanes with a short-carbon chain (up to six carbons) proceeds via hydrolytic mechanism, whereas long carbon chain haloalkanes are dehalogenated oxidatively (Fetzner and Lingens, 1994). Haloalkane dehalogenases originating from various bacterial strains have been described (Scholtz et al., 1987a; Janssen et al., 1988; Sallis et al., 1990; Van den Wijngaard et al., 1992; Nagata et al., 1993b; Poelarends et al., 1999; Jesenska et al., 2005; Sato et al., 2005; Sfetsas et al., 2009).

1.3.1 Classification of haloalkane dehalogenases

Previously, haloalkane dehalogenases (HLDs) were classified according to their substrate specificity and four classes of HLDs were proposed, represented by DhlA, LinB, DhaA, and DbjA haloalkane dehalogenases (Damborsky et al., 1997). Damborsky et al. (2001) conducted structure-specificty relationship study of haloalkane dehalogenases and identified 3 classes with different structural role and substrate specificity: class I; represented by DhlA of *Xanthobacter autotrophicus*

GJ10 (Janssen et al., 1985; Janssen et al., 1988), class II; represented by DbjA of *Bradyrhizobium japonicum* (Sfetsas et al., 2009) and DhaA of *Rhodococcus erythroplis* Y2 (Sallis et al., 1990), and class III; represented by LinB of *Sphigobium japonicum* UT26 (Nagata et al., 1993a; Nagata et al., 2006).

Recently, phylogenetic analysis of haloalkane dehalogenases family (HLDs) revealed existence of three subfamilies of HLDs designated HLD-I, HLD-II, HLD-III (Chovancova et al., 2007). The three of the former classes distinguished by substrate specificity (DhaA, LinB, and DbjA) belonged to the same subfamily (HLD-II) in the phlogenetic based classification (Chovancova et al., 2007). Chovancova et al. (2007), Prudnikova et al. (2009) and Sfetsas et al. (2009) reported representatives of these haloalkane dehalogenase subfamilies: HLD-I subfamily is represented by DhlA of *Xanthobacter autotrophicus* GJ10; (Keuning et al., 1985; Janssen et al., 1988), DhmA of *Mycobacterium avium*; (Jesenska et al., 2002), DmbB of *Mycobacterium tuberculosis* (Jesenska et al., 2005). HLD-II subfamily is represented by DbjA of *Bradyrhizobium japonicum* and DmlA of *Mesorhizobium loti*; (Sato et al., 2005; Sfetsas et al., 2009), DmbA of *Mycobacterium tuberculosis*; (Jesenska et al., 2005), DhaA of *Rhodococcus rhodochrous* strain NCIMB13064; (Curragh et al., 1994), LinB of *Sphigobium japonicum* UT26 (Nagata et al., 1993a; Nagata et al., 2006), DbeA of *Bradyrhizobium elkani*; (Prudnikova et al., 2009). HLD-III subfamily is represented by DrbA of *Rhodopirellula baltica* SH1, and DmbC of *Mycobacterium tuberculosis* (Jesenska et al., 2009).

1.3.2 Reaction mechanism of haloalkane dehalogenases

Janssen at al. (1985) first described the hydrolytic haloalkane dehalogenase (DhlA) from *Xanthobacter autotrophicus* GJ10, which utilizes 1,2-dichloroethane as sole source of carbon and energy (Janssen et al., 1985; Keuning et al., 1985; Janssen et al., 1988). DhlA is one of the well studied dehalogenase, and has been purified (Keuning et al., 1985), crystallized and its three-dimentional structure was determined (Franken et al., 1991; Verschueren et al., 1993b).

The reaction mechanism of the haloalkane, DhlA, of *Xanthobacter autotrophicus* GJ10 (Figure 1.2), determined by X-ray crystallography and site-directed mutagenesis, indicated that the dehalogenation of 1, 2-dichloroethane is achieved by

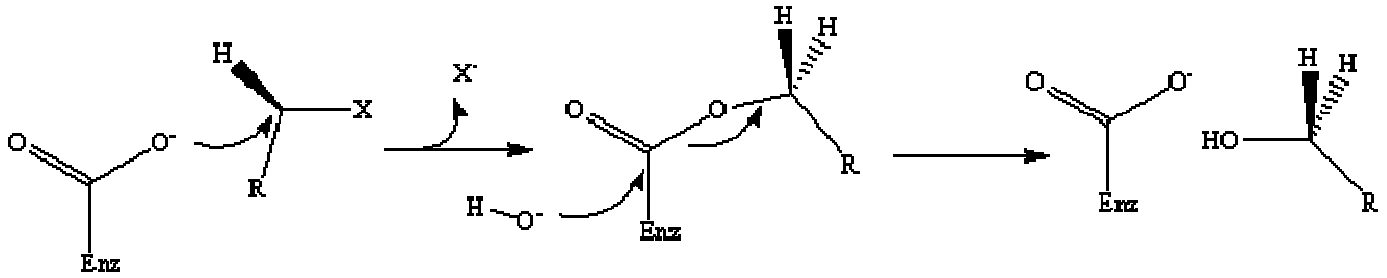

Figure 1.2 Hydrolytic mechanism of dehalogenation of haloalkane compounds (Bohac et al., 2002). R represents an alkyl group, X represents a halogen atom.

by two step catalytic mechanism. The reaction initiates by a nucleophilic attack ($Asp_{124)}$ on the halogen-carbon bond of the substrate, leading to the formation of covalent alkyl-enzyme intermediate which subsequently is cleaved by a water molecule activated by the base His_{289} (Verschueren et al., 1993a; Verschueren et al., 1993b; Verschueren et al., 1993c; Marek et al., 2000; Prokop et al., 2003). There is no evidence indicating the involvement of co-factor or metal ions in the catalytic mechanism (Janssen et al., 1994b; Damborsky and Koca, 1999).

DhlA of strain GJ10, dechlorinates 1,2-dichloroethane to 2-chloroethanol, which is then converted by an alcohol dehydrogenase (encoded by *mox*) to 2-chloroacetaldehyde, which in turn is converted to MCA by an aldehyde dehydrogenase (encoded by *ald*) (Figure 1.3) (Janssen et al., 1989). MCA is dechlorinated to glycolate, which will enter central metabolism, by an α-haloacid dehalogenase, DhlB (Figure 1.3) (Janssen et al., 1989; Tardif et al., 1991). DhlA was also able to catalyze dehalogenation of C1-C4 α-halogenated n-alkane and α,ω-dihalogenated n-alkane, including chlorinated, brominated, and iodinated compounds (Keuning et al., 1985; Damborsky and Koca, 1999).

The reaction mechanism of DhlA of strain GJ10 was compared with DhaA of *Rhodococcus rhodochrous* (Newman et al., 1999), and LinB of *Sphingobium japonicum* UT26; (Marek et al., 2000; Oakley et al., 2004), and it was found that their sequences, structures and kinetic mechanisms were all similar (Prokop et al., 2003), (Damborsky and Koca (1999). The haloacid dehalogenase DehH1 of *Delftia acidovorans* strain B (Formerly *Moraxella* sp. strain B) (Kawasaki et al., 1981a), which defluorinates fluoroacetate showed considerable similarity in structure and catalytic mechanism with the haloalkane dehalogenases (Kawasaki et al., 1992; Krooshof et al., 1997).

1.3.3 Involvement of HLDs in catabolic pathways for degradation of haloalkanes

The microbial host diversity of haloalkane dehalogenases is broader compared with haloacid (αHA) dehalogenases. Haloalkane dehalogenases have been found in Gram-negative (Janssen et al., 1985) as well as Gram-positive genera (Scholtz et al., 1987a; Yokota et al., 1987; Sallis et al., 1990; Olaniran et al., 2004). Moreover, haloalkane dehalogenases exhibit broader substrate specificity. Keuning et al. (1985) isolated

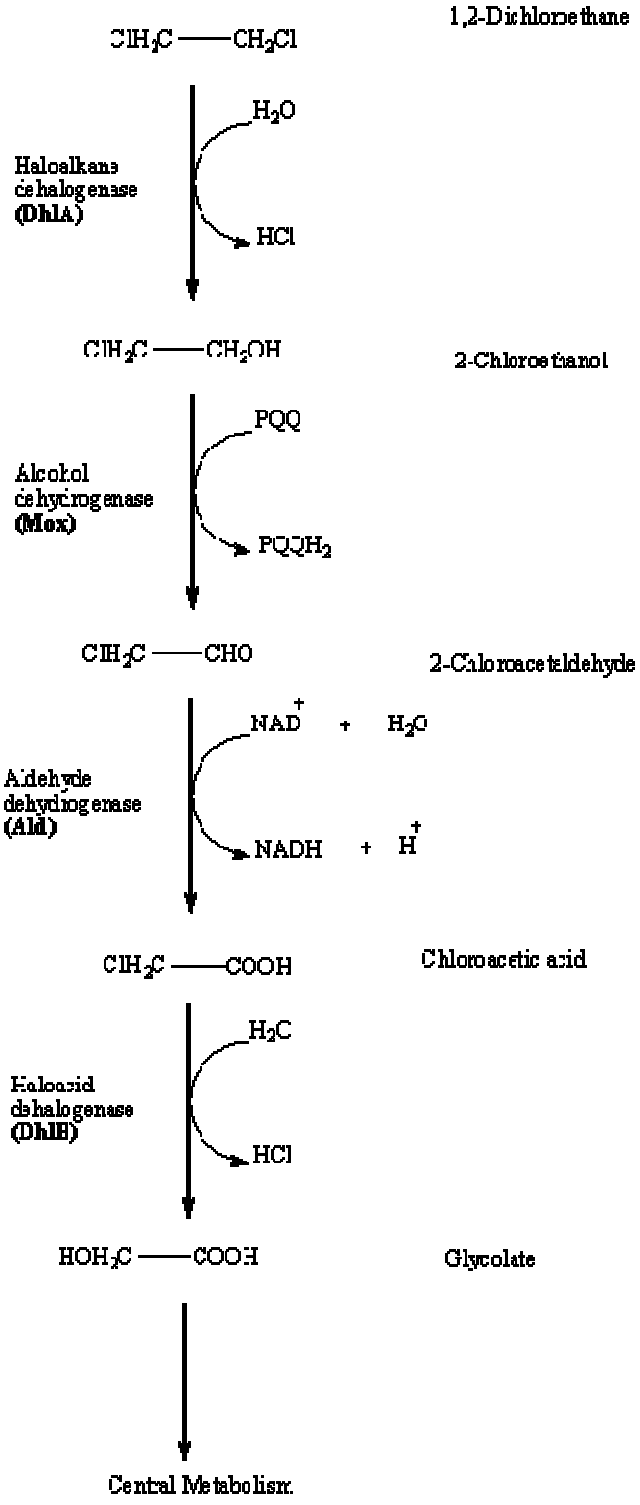

Figure 1.3 Biodegradation pathway for 1,2-dichloroethane in *Xanthobacterium autotrophicus* GJ10. DhlA, haloalkane dehalogenase; Mox, alcohol dehydrogenase; Ald, aldehyde dehydrogenase; DhlB, haloalacid dehalogenase; PQQ = pyrroquinoline quinone (Janssen et al., 1989).

Xanthobacter autotrophicus GJ10 which dehalogenates chlorinated, brominated, and iodinated α-substituent C1 and C4 compounds. Scholtz et al. (1987b) showed that *Arthrobacter* strain HA1 (Jesenska et al., 2000) could utilize over 18 C2 to C8 α-halogenated compounds as growth substrates and also dehalogenated other compounds, including dichlorinated alkanes. *Rhodococcus rhodochrous* strain NCIMB13064 degrades 1-chlorobutane and produces a hydrolytic haloalkane dehalogenase (also designated DhaA) that can utilize a wide range of 1-haloalkanes as sole carbon and energy sources, but C5 and C8 bromoalkanes are not growth substrate (non-inducer), even though they are debrominated by the dehalogenase (DhaA). The haloacids such as 2MCPA and MCA are not substrates for the DhaA enzyme (Curragh et al., 1994; Damborsky et al., 1997; Newman et al., 1999). The wide haloalkane substrate range of strain NCIMB13064 is similar to that reported for strain Y2 by Sallis et al. (1990), which grows on C14, C16, C18 alkanes, but does not show any short chain haloalkane dehalogense activity (Curragh et al., 1994). Amino acid sequences comprisons indicated that the DhaA of strain NCIMB13064 significantly resembled the haloalkane dehalogenase, $DhaA_f$ of *Mycobacterium* sp. GP1, which degrades 1,2-dibromoethane and is constitutively produced (Kulakova et al., 1997; Poelarends et al., 1999). A hydrolytic haloalkane dehalogenase from *Pseudomonas pavonaceae* strain 170 (Poelarends et al., 1998) involved in degradation of nematocide 1,3-dichloropropene was also 100% identical to DhaA of strain NCIMB13064 (Poelarends et al., 1998; Poelarends et al., 1999). In contrast to the inducible production of DhaA by strain NCIMB13064, DhaA is constitutively produced in strain 170. Comparisons between DhlA of *Xanthobacter autotrophicus* GJ10 and DhaA of strain NCIMB13064 (32% sequence identity) showed that DhaA has a larger substrate-binding cavity (Newman et al., 1999; Bohac et al., 2002), and differed in terms of halide release kinetics (Schindler et al., 1999).

Sphingobium japonicum strain UT26 (Nagata et al., 2006) utilizes the insecticide γ-hexachlorocyclohexane (γ-HCH or lindane) via hydrolytic dehalogenation by tetrachlorocyclohexadiene dehalogenase, LinB, which is constitutively expressed (Nagata et al., 1993a; Nagata et al., 1997; Nagata et al., 1999; Nagata et al., 2005). LinB catalyzes the conversion of 1,3,4,6-tetrachloro-1,4-cyclo-hexadiene via two hydrolytic steps to 2,5-dichloro-2,5-cyclohexadiene-1,4-diol via 2,4,5-trichloro-2,5-cyclohexane-1-ol (Nagata et al., 1993a). This dehalogenase has a broad range

substrate specificity and is able to act on monochloroalkanes (C3-C10), dichloralkanes, bromoalkanes, and chlorinated aliphatic alcohols. LinB is more similar to DhaA (49% sequence identity) than to the DhlA (30% identity) (Kahn and Bruice, 2003). LinB and DhaA can dehalogenate compounds with chain lengths up to 10 carbon atoms, but they can not catalyze the dehalogenation of 1,2-dichloroethane (Damborsky and Koca, 1999). LinB, DhlA, and DhaA have significant amino acid sequence similarity with each other, and also with the fluoroacetate-specific, H-1 dehalogenase of *Delftia acidovorans* strain B (formerly *Moraxella* sp. strain B) (Krooshof et al., 1997).

Some haloalkane-degraders produce other dehalogenases. For example, *Xanthobacter autotrophicus* GJ10 and *Xanthobacter flavus* UE15, both produce haloalkane (designated DhlA) and haloacids (designated DhlB) dehalogenases (Janseen et al. 1985(Song et al., 2003), encoded by *dhlA* and *dhlB*, respectively (Song et al., 2004). The amino acid sequences of the DhlA of *Xanthobacter flavus* UE15 is identical to the DhlA of *Xanthobacter autotrophicus* GJ10, but only 27-30% sequence identity with the corresponding enzymes from *Rhodococcus rhodochrous* (Kulakova et al., 1997), *Mycobacterium* GP1 (Poelarends et al., 1999), and *Pseudomonas pavonaceae* 170 (Poelarends et al., 1998). *Rhodococcus erythropolis* Y2, isolated from soil by enrichment with 1-chlorobutane (Sallis et al., 1990), also produces two types of dehalogenases: a hydrolytic dehalogenase (DhaA) induced by C3-C16 1-haloalkane substrates, and oxygenase, induced by C7-C16 1-haloalkanes (Armfield et al., 1995). *Sphingobium japonicum* strain UT26 produces LinB which is involve in degradation of the insecticide γ-HCH (lindane) via hydrolytic dehalogenation, and γ-HCH dehydrochlorinase dehalogenase enzymes involved in catalyzing γ-HCH purified from strain UT26 (Nagata et al., 2006), such as LinA and the reductive LinD (Imai et al., 1991; Nagata et al., 1999; Nagata et al., 2007). A *Rhodococcus* sp. producing a haloalkane dehalogenase (Scholtz et al., 1988a), and a β-chloropropionate halidohydrolase has also been reported (Scholtz et al., 1987a; Armfield et al., 1995).

1.4 Haloacid dehalogenases (αHA Deh)

The αHA dehalogenases have been intensively studied with respect to their phylogenetic relationships, structure and reaction mechanisms. The αHA

dehalogenases catalyze the conversion of α-haloacids (αHAs) to hydroxyhaloacids by hydrolytic dehalogenation (Figure 1.4; Fetzner and Lingens, 1994).

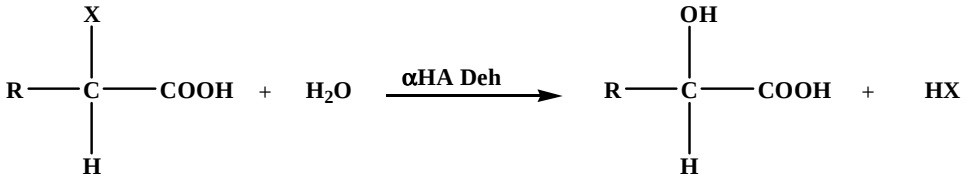

Figure 1.4 Hydrolytic mechanism of dehalogenation of haloacid compounds. Halogen substitute is replaced in nucleophilic substitution reaction by hydroxyl. R represents an alkyl group, X represents a halogen atom.

Table 1.1 shows a number of the characterized α-HA dehalogenases active towards chlorinated compounds.

1.4.1 Classification of α-HA dehalogenases

Initial attempts to classify αHA dehalogenases were based on differences in the enzyme activities. For example, four classes of αHA dehalogenases were proposed by Slater et al. (1997) according to substrate specificities and stereo-selectivity, electrophoretic mobility and molecular weight. This method of classification has been replaced by molecular phylogenetic methods (Hill et al., 1999), based on dehalogenase gene sequences, which divided αHA dehalogenases into two main families: DehI (or group I, Figure 1.5A) and DehII (or group II, Figure 1.5B). Group I and Group II dehalogenases catalyse dehalogenation by fundamentally different mechanisms, indicating that the two dehalogenases genes are not evolutionary related (Hill et al., 1999). Both groups are active with organic acids of low molecular weight where the halogen located at the C2-(or α-) position, but they differ in their stereoselectivity and their mode of hydrolytic attack (Schmidberger et al., 2008).

Group I dehalogenases are phylogenetically distinct from Group II and have no other close evolutionary relatives (Figure 1.5A), and show broader stereoselectivity than the

Table 1.1 Gene designations and substrate specificities of cloned haloacid dehalogenases

Bacterial strain	Dehalogenase[a]	Gene designation	Substrate used for isolation	Substrate							Reference
				MCA	DCA	2MCPA	2,2DCPA	2,2DCBA	MBA	2MBPA	
Pseudomonas sp. strain CBS3	DehCI (II)	*dehCI*	4-CBA	+	+	+	nd	nd	+	+	(Schneider et al., 1991)
Pseudomonas sp. strain CBS3	DehCII (II)	*dehCII*	4-CBA	+	+	+	nd	nd	+	+	(Schneider et al., 1991)
Pseudomonas putida AJ1	HadD (I)	*hadD*	2MCPA	+	nd	+	+	nd	+	+	(Barth et al., 1992)
Pseudomonas putida AJ1	HadL (II)	*hadL*	2MCPA	nd	nd	+	nd	+	nd	+	(Jones et al., 1992)
Pseudomonas putida PP3	DehI (I)	*dehI*	2MCPA	+	+	+	+	nd	nd	nd	(Thomas et al., 1992b)
Pseudomonas putida PP3	DehII (II)	*dehII*	2,2DCPA	+	+	+	+	nd	nd	nd	(Weightman et al., 2002)
Pseudomonas putida No. 109	H-109 (II)	*dehH109*	2MCPA	+	+	+	+	nd	+	+	(Motosugi et al., 1982c; Kawasaki et al., 1994)
Pseudomonas sp. strain 113	DL-DEX (I)	*DL-DEX*	2MCPA	+	+	+	+	nd	+	+	(Motosugi et al., 1982b; Nardi-Dei et al., 1997)
Pseudomonas sp. strain YL	L-DEX YL (II)	*L-DEX YL*	2MCPA	+	nd	+	+	nd	+	nd	(Liu et al., 1994)
Pseudomonas sp. strain YL	DL-DEX YL (II)	*DL-DEX YL*	2-CAA	+	nd	+	+	nd	+	nd	(Nardi-Dei et al., 1994)
Burkholderia cepacia MBA4	Deh4a (II)	*Deh4a*	MBA	+	_	+	nd	nd	+	+	(Tsang and Pang, 2000)
Rhizobium sp. RC1	DehL (NA)	*dehL*	22DCPA	nd	+	+	nd	nd	nd	nd	(Cairns et al., 1996)

Continued

Rhizobium sp. RC1	DehE (I)	*dehE*	22DCPA	+	+	+	+	nd	nd	nd	(Stringfellow et al., 1997)
Rhizobium sp. RC1	DehD (I)	*dehD*	22DCPA	+	+	+	+	nd	nd	nd	(Cairns et al., 1996)
Azotobacter sp. strain RC26	(NA)	NA	MCA	+	+	+	+	nd	nd	nd	(Diez et al., 1996)
Xanthobacter autotrophicus GJ10	DehB (II)	*dehB*	2MCPA	+	+	+	nd	nd	nd	nd	(Ridder et al., 1997)
Delftia acidovorans strain B	H-2 (II)	*dehH2*	MCA	+	nd	nd	nd	nd	nd	nd	(Sota et al., 2002)
Methylobacterium sp. HJ1	DehE (I)	*dehE*	2,2DCPA	+	+	+	+	+	nd	nd	(Jing et al., 2008)
Alcaligenes xylosoxidans spp. *denitrificans* ABIV	DhlIV (I)	*dhlIV*	DCA	+	+	+	+	nd	+	nd	(Brokamp et al., 1996)

a. Dehalogenase gene family shown, where known, in brackets, as described by Hill et al. (1999)

+ = activity, - = no activity , nd = not determined

Abbreviation as follows: 4CBA – 4 chlorobenzoate, 2CAA – 2 chloroacrylate, MCA – monochloroacetic acid, DCA– Dichloroacetic acid, 2MCPA – 2 monochloropropionic acid, 22DCPA – 2, 2 dichloropropionic acid, 22DCBA – 2, 2 dichlorobutric acid, MBA – monobromoacetic acid, 2MBPA – 2 monobromopropionic acid
NA denotes no information available

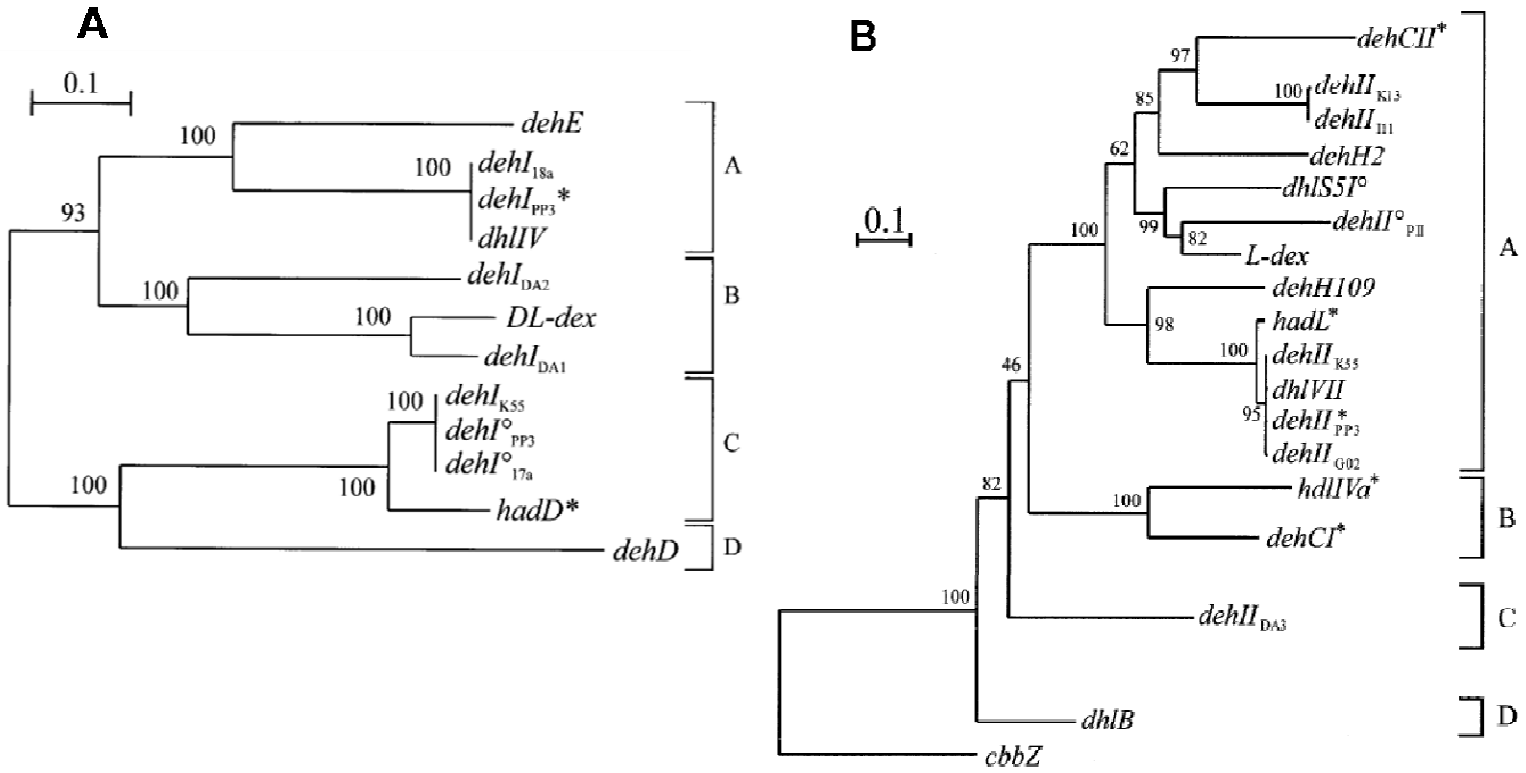

Figure 1.5 Phylogenetic trees illustrating the relationship between previously characterized Group I (A, *dehI*) and Group II (B, *dehII*) α-HA dehalogenase genes (*deh*) with four subgroups each (Hill et al., 1999). Subscripts indicate bacterial strains contained the *deh* gene, the asterisks (*) indicate *deh* used for PCR primers designing, the mark (^{o}) indicate silent *deh* gene. The gene abbreviation used were *dehE* (Stringfellow et al., 1997); $dehI_{PP3}$ (Thomas et al., 1992b); *dhlIV* (Brokamp et al., 1996); *DL-dex* (Nardi-Dei et al., 1997; Laturnus et al., 2005); hadD and hadL (Jones et al., 1992; Gribble, 1996; Teuten and Reddy, 2007); *dehD* (Cairns et al., 1996); *dehCI* and *dehCII* (Schneider et al., 1991); *dehH2* (Kawasaki et al., 1992); $dhlS5I^{o}$ (Köhler et al., 1998); *L-dex* (Nardi-Dei et al., 1994); *dehH109* (Kawasaki et al., 1994); *dhlVII* (*P. fluorescens* ABVII; Honnens et al., unpublished); *hdlIVa* (Murdiyatmo et al., 1992); *dhlB* (Van der Ploeg et al., 1991); *cbbZ* (Schaferjohann et al., 1993); and $dehI_{18a}$ (*Pseudomonas* sp.), $dehI_{DA2}$ (*Bradyrhizobium* sp.), $dehI_{DA1}$ (*Burkholderia* sp.), $dehI_{K55}$ (*Pseudomonas* sp.), $dehI^{o}_{PP3}$ (*Pseudomonas putida* PP3), $dehI^{o}_{17a}$ (*Pseudomonas* sp.), $dehII_{K13}$ (*Burkholderia* sp.), $dehII_{I11}$ (*Burkholderia* sp.), $dehII^{o}_{PII}$ (*Burkholderia* sp.), $dehII_{K55}$ (*Pseudomonas* sp.), $dehII_{PP3}$ (*Pseudomonas putida* PP3), $dehII_{G02}$ (*Burkholderia* sp.), and $dehII_{DA3}$ (*Bradyrhizobium* sp.), *cbbz*, a 2 phosphoglucolate from *Alkaligenes eutrophus*, a member of the HAD superfamily (Hill et al., 1999).

group II dehalogenases. Some group I αHA dehalogenases are able to catalyze dehalogenation of both L- and D-haloacids, whereas others show activity with D-haloacids only (Weightman et al., 1982; Barth et al., 1992; Hill et al., 1999; Schmidberger et al., 2008). The DehI subgroups C and D contain group I dehalogenases acting on D-haloacids, while the dehI subgroups A and B (Figure 1.5A) contain dehalogenases active on both D- and L- haloacids. Dehalogenation by group I αHA dehalogenases involves a direct attack of an activated water molecule on the C2 carbon of the haloacids without formation of enzyme-substrate intermediates (Nardi-Dei et al., 1999).

Group II dehalogenases are stereoselective, showing activity exclusively with L-haloacids but not D-haloacids. Koonin and Tatusov (1994) analyzed bacterial haloacid dehalogenases and showed that they belong to a large superfamily called haloacid dehalogenase (HAD) superfamily, which also include some ATPases, epoxide hydrolases and a number of different phosphatases. Like haloalkane dehalogenases, the group II haloacid dehalogenases use covalent catalysis. The mechanism action of DehII studied by X-ray crystalography showed that the reaction involves a nucleophilic attack of the C2 carbon, forming an esterified enzyme-substrate intermediate (Ridder et al., 1999).

There are probably other αHA dehalogenases families. For example, *dehH1* gene from *Delftia* sp. strain B different from group I and group II dehalogenases, encodes a defluorinating dehalogenase with broader substrate specificity acting on haloalkane (Kawasaki et al., 1992), and *dehL* from *Rhizobium* sp. showed the same stereospecificity as group II *deh* genes acting only on L-2MCPA, but no significant match in nucleotide or amino acid sequences with group II *deh* genes was found (Cairns et al., 1996). These genes were phylogenetically unrelated to any *deh* genes which have been described by Hill et al. (1999).

Pseudomonas putida PP3, which evolved the ability to utilize αHAs during continuous-flow enrichment culture of strain PP1 in the presence of the herbicide 22DCPA (Senior et al., 1976), produces two α-HA dehalogenases, DehI and DehII encoded by *dehI* and *dehII*, respectively (Weightman et al., 1979).

1.4.2 Dehalogenation mechanism of Group I αHA dehalogenases

The catalytic mechanism of Group I dehalogenases was originally proposed for DL-DEX from *Pseudomonas* sp. strain 113. Results from site-directed mutagenesis, ^{18}O-incorporation, and crystal structure studies of DL-DEX (Motosugi et al., 1982b) suggested a single step mechanism, and that substrate hydrolysis proceeds through a direct attack of water molecule on the C2 carbon of the haloacid substrates, displacing the halogen atom, but not via a covalent ester intermediate (Nardi-Dei et al., 1997; Nardi-Dei et al., 1999; Kurihara et al., 2000; Papajak et al., 2006). This single-step mechanism was consistent with that originally proposed by Goldman et al. (1968). Group II dehalogenases have been found in several bacteria such as *Pseudomonas putida* PP3 (Table 1.1). Recently, Schmidberger et al. (2008) reported the crystal structure of DehI from *Pseudomonas putida* PP3, and showed the enzyme was able to process both L- and D-haloacids with inversion of configuration to D- and L-hydroxyl acids products, respectively. They also showed that the proposed reaction mechanism of DehI was consistent with that of DL-DEX of *Pseudomonas* sp. strain 113, as proposed by Nardi-Dei et al. (1999) (Figure 1.6A). The study suggested that stereoselectivity of the D-haloacid dehalogenases is due to substitution of a single amino acid residue (Ala_{187} Asn) preventing binding of the L-haloacids (Schmidberger et al., 2008).

1.4.3 Dehalogenation mechanism of Group II αHA dehalogenases

Most αHA dehalogenases studied to date are Group II and, therefore, L-specific (Hill et al., 1999). The mechanism of group II αHA dehalogenases has been analyzed by studies of L-DEX from *Pseudomonas* sp. YL, and is similar to that of haloalkane dehalogenase (section 1.3.2), in that dehalogenation is achieved by a nucleophilic attack of the haloacid C2 carbon by an aspartate residue, Asp_{10}, forming an esterified enzyme-substrate intermediate, which is subsequently cleaved by a second nucleophilic attack by an activated water molecule, yielding the free enzyme and product (Figure 1.6B) (Liu et al., 1995; Li et al., 1998; Ridder et al., 1999; Schmidberger et al., 2007; Kurihara and Esaki, 2008). This reaction is also similar to the reaction catalyzed by fluoroacetate dehalogenase DehH-1 of *Delftia acidovorans* strain B (Kawasaki et al., 1992; Krooshof et al., 1997).

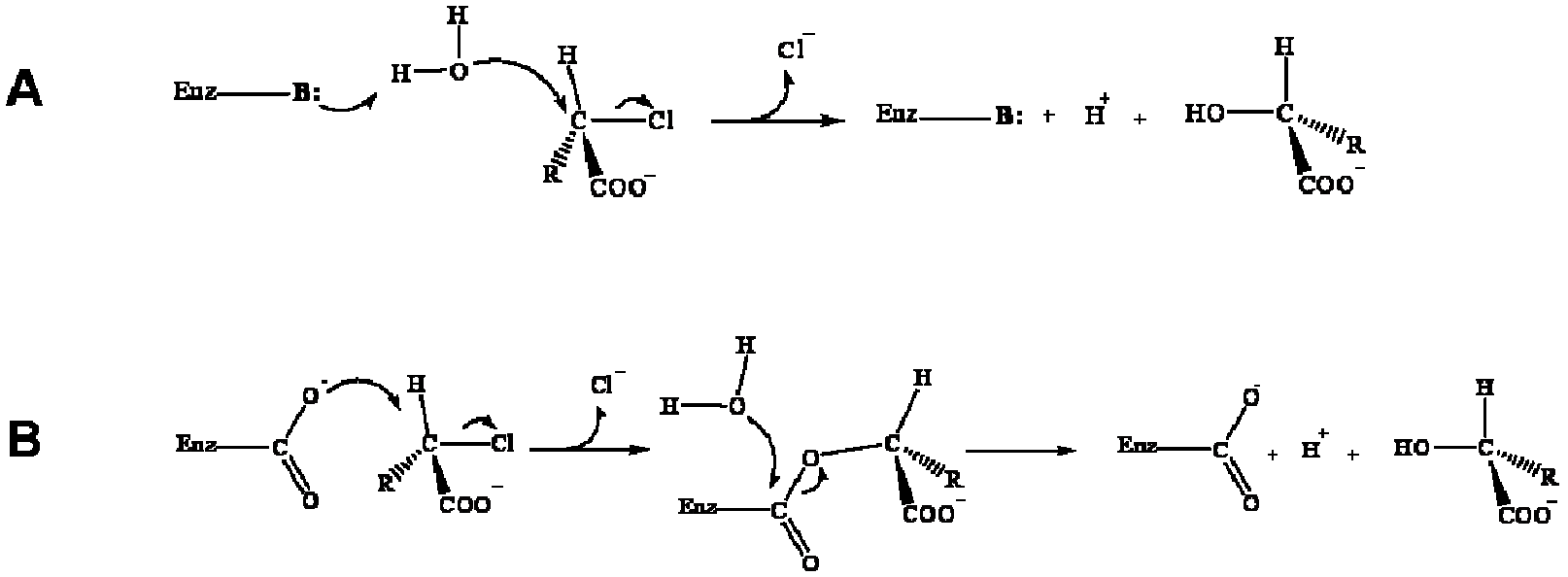

Figure 1.6 Reaction mechanisms for hydrolytic dehalogenation of 2-haloacids by DehI and DehII enzymes. (A) For DehI enzymes, reaction mechanism involving direct nucleophilic attack of water on the halogenated compound displacing the halogen. The reaction of DL-2-haloacid dehalogenase proceeds by this mechanism. L-2-Haloacid is shown as the substrate here, but D-2-haloalkanoic acid also serves as a substrate for DL-2-haloacid dehalogenase (Kurihara and Esaki, 2008). (B) For DehII enzymes, the halogenated compound undergoes a nucleophilic attack of an active site carboxylate group resulting in a halogen removal and formation of an ester intermediate which then hydrolysed by an enzyme activated water molecule. The reaction of L-2-haloacid dehalogenase proceeds by this mechanism. R represents an alkyl group, X represents a halogen atom.

Liu et al. (1994) and Nardi-Dei et al. (1994), also studying L-DEX from strain YL, found that the enzyme was thermostable, composed of two identical subunits and inducible. The enzyme acts on short carbon chain length 2-haloacids such as chloroacetate (MCA) and L-2-chloropropionate (2MCPA), as well as long carbon chain 2-haloacids such as 2-bromohexadecanoate (Liu et al., 1994). The catalytic role of Asp_{10} in L-DEX was confirmed by replacement Asp_{10} by other amino acids (Ala, Gly, Ser or Glu) which resulted in its total inactivation. However, replacement of Asp_{10} by Asn did not completely inactivate the enzyme (Kurihara et al., 1995). The studies of the enzyme by a combination of ^{18}O-incorporation, mass spectrometry of labelled enzyme and x-ray crystallography indicated that an oxygen atom of the solvent water was first incorporated into the enzyme and then transferred to the product (Liu et al., 1995; Hisano et al., 1996; Liu et al., 1998). The crystal structure of L-2-haloacid dehalogenases from other bacteria have also been reported, such as DhlB of *Xanthobacter autotrophicus* GJ10 (Ridder et al., 1997; Ridder et al., 1999), and HdlIVa (Yu et al., 2007) of *Burkholderia cepacia* MBA4 (Schmidberger et al., 2007).

1.4.4 Dehalogenation mechanism of fluoroacetate-specific dehalogenases

Fluoroacetate dehalogenase catalyzes the hydrolytic dehalogenation of fluoroacetae and other haloacetates to produce glycollate and F^- (Liu et al., 1998; Kurihara et al., 2003), and was first reported in a *Pseudomonas* sp. by Goldman (1965). *Delftia acidovorans* strain B (formerly *Moraxella* strain B) produces two haloacetate dehalogenases one of which, H-1 (FAc-DEX H1) is fluoroacetate-specific, whereas H-2 acts on the haloacetate but can not dechlorinate fluoroacetate ((Kawasaki et al., 1981a). Liu et al. (1998) proposed a catalytic mechanism for fluoroacetate dehalogenase H-1 where the carboxylate group of Asp105 acts as a nucleophile attacking the α-carbon atom of fluoroacetate to displace the fluorine atom, leading to the formation of ester intermediate which is hydrolysed by a water molecule activated by His_{272} (Liu et al., 1998; Kurihara et al., 2000). An ester intermediate is produced, similar to that of L-2-haloacid dehalogenase, although there is no sequence similarity between the two enzymes (Liu et al., 1998). Fluoroacetate-specific H-1 is structurally similar to haloalkane dehalogenase DhlA of *Xanthobacter autotrophicus* GJ10 (Franken et al., 1991; Liu et al., 1998). Despite this sequence similarity, fluoroacetate

dehalogenase is different from haloalkane dehalogenase in substrate specificity (Kurihara et al., 2000).

Burkholderia sp. FA1 also produces a fluoroacatete-specific dehalogenase, FAc-DEX FA1 which has been cloned and sequenced (Kurihara et al., 2003). Recently, the three-dimensional structure of FAc-DEX FA1 was determined and the reaction mechanism of the enzyme was proposed to proceed via the two-step mechanism (Figure 1.7; Jitsumori et al., 2009). Fluoroacatete-specific FA1 (FAc-DEX FA1) of *Burkholderia* sp. FA1 was structurally and functionally related to H-1 (FAc-DEX H1) of *Delftia acidovorans* strain B (Jitsumori et al., 2009), and also appears to be a member of the haloalkane dehalogenase family (section 1.3.1).

1.4.5 Involvement of αHA dehalogenases in catabolic pathways for degradation of αHAs

αHA degrading bacteria have been isolated from contaminated (Janssen et al., 1985; Kawasaki et al., 1992; Hill and Weightman, 2003) and pristine sites (Klages et al., 1983; Marchesi and Weightman, 2003a). The occurrence of dehalogenases in these strains has been described, and in all cases the αHA degraders were members of *Proteobacteria* and in particular α, β, and γ sub-phyla (Hill and Weightman, 2003; Marchesi and Weightman, 2003b, a), such as *Pseudomonas*, *Xanthobacter*, and *Burkholderia* (see Table 1.1). However, the diversity of αHA degraders also includes Gram-positive genera, such as *Bacillus* and *Enterococcus* (Kerr and Marchesi, 2006).

Several bacterial strains degrading αHA compounds have been reported to contain more than one dehalogense (see Table 1.1) (Goldman et al., 1968; Weightman et al., 1979; Weightman et al., 1982; Leigh et al., 1988). For example, when growing on 2MCPA *Pseudomonas putida* PP3 produces DehI and DehII, and two stereospecific haloacid dehalogenase (HadD, HadL) are produced by *Pseudomonas putida* AJ1 (Smith et al., 1990; Barth et al., 1992; Jones et al., 1992). *Rhizobium* sp. strain RC1 also produces two haloacid dehalogenases of opposite stereospecificities: DehD, specific toward D-haloacids; and DehL, specific toward L-haloacids

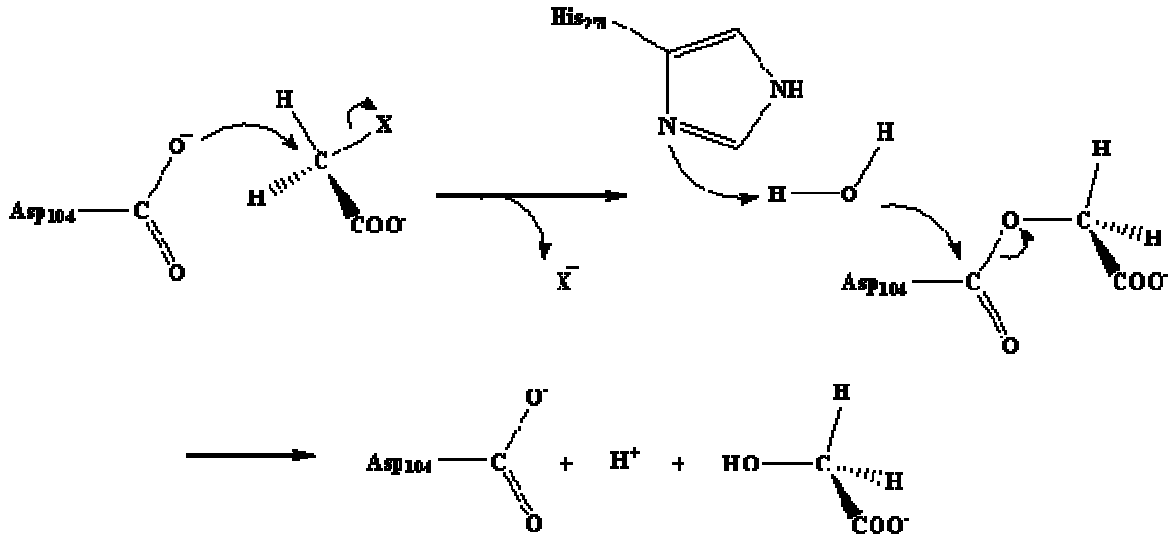

Figure 1.7 Proposed reaction mechanism of FAc-DEX FA1 dehalogenase of *Burkholderia* sp. FA1. The halogenated compound undergoes a nucleophilic attack by an active cite carboxylate group, resulting in halogen removal and formation of an ester intermediate which is then hydrolyzed by an activated water molecule. X represents a halogen atom; R represents a hydrogen atom or an alkyl group (Jitsumori et al., 2009).

(Berry et al., 1979; Allison et al., 1983; Cairns et al., 1996; Huyop and Cooper, 2003; Huyop et al., 2008), as well as a third non-stereosepecific dehalogenase, DehE, active with D- and L-haloacids (Leigh et al., 1988; Stringfellow et al., 1997; Huyop et al., 2004). *Pseudomonas* sp. CBS3 isolated on 4-chlorobenzoate, was reported to produce two types of dehalogenases; oxygenase dehalogenases, and also hydrolytic αHA dehalogenases (DehCI and DehCII) induced by 4-chlorobenzoate, both were active toward L-2MCPA (Klages et al., 1983; Schneider et al., 1991). *Xanthobacter autotrophicus* GJ10, besides producing the αHA dehalogenase (DhlB), also contains a second dehalogenase, DhlA acts on haloalkane dehalogenases, 1,2-dichloroacetic acid (section 1.3.2, Figure 1.8) (Janssen et al., 1985).

Transport of αHAs into the bacterial cell, where degradative enzymes act, is an important part of catabolism of these compounds. Previous studies on the toxicity of haloacids to *Pseudomonas putida* strain PP3 suggested the presence of dehalogenase-associated permease (Weightman et al., 1985). Permease genes encoding a Na^+/H^+ symport transporter involved in the uptake of haloacids have been identified in *Pseudomonas putida* strain PP3 (Dodds, 2003). A similar genomic structure was also found in *Agrobacterium* strain NHG3, where putative transporter gene, *dehp* was located upstream of D-specific and L-specific 2MCPA dehalogenases (Higgins et al., 2005); however, the function of this putative transporter was not investigated. In *Xanthobacter autotrophicus* strain GJ10, the protein DhlC appears to have a role as a haloacids transporter (Van der Ploeg et al., 1995), and the *dhlC* gene was reported to be located chromosomally within a cluster of related genes. Mutants lacking the region of DNA containing this gene were unable to grow on αHAs. More recently, a dehalogenase-associated permease (*deh4p*) was identified and characterized in *Burkholderia cepacia* MBA4 located downstream of *deh4a* encodes a trans-membrane protein responsible for the uptake of haloacetate (Yu et al., 2007).

1.5 Genetic analysis of bacterial degradation of haloalkane and halocacids

The first αHA dehalogenase genes to be cloned and sequenced were *dehCI* and *dehCII* of *Pseudomonas* sp. strain CBS3 (Schneider et al., 1991). Since then several group I and II αHA *deh* genes, and genomic regions, have been sequenced (Table 1.1; Figure 1.9). Several bacteria are known to carry the genes for biodegradation of

chlorinated compounds on mobile genetic elements, such as plasmids (Motosugi et al., 1982c), or transposable elements on the chromosome (Slater et al., 1985; Weightman et al., 2002), and therefore have the potential for horizontal gene transfer (HGT) (Kawasaki et al., 1992; Van der Meer et al., 1992; Kawasaki et al., 1994). Transposons and insertion sequences (IS) encode transposases that catalyze transposition, and they can move by recombination from one genetic site to another (Mahillon and Chandler, 1998; Top and Springael, 2003; Mijnendonckx et al., 2011). Gene mobilization between and within genomes plays an important role in genetic adaptation to degrade xenobiotics (Janssen et al., 2005). Transposons have been identified that are associated with dehalogenases; for example, IS*1071* located near *dehH2* in *Moraxella* sp. strain B on pUO1 plasmid (Kawasaki et al., 1992) was also found close to haloalkane *deh* gene (*dhaA*) of *Pseudomonas pavonaceae* strain 170 (Poelarends et al., 1998). Evidence has also been reported to suggest that activation and or silencing dehalogenase genes is associated with transposition of insertion sequences (Weightman et al., 2002).

Isolation and genetic characterization of bacteria from different parts of the world containing haloalkane and lindane (γ-HCH) associated dehalogenases has provided strong evidence for HGT of the genes encoding these enzymes (Poelarends et al., 2000a; Dogra et al., 2004; Chovancova et al., 2007; Nagata et al., 2007).

1.5.1 The organization of hakoalkane dehalogenase genes

The genes *dhaA*, *adhA* and *aldA* which encode enzymes responsible for the degradation of 1-chlorobutane in *Rhodococcus rhodochrous* strain NCIMB13064 are clustered with the putative *dhaA* regulatory gene *dhaR* (Figure 1.8) on the host chromosome and on the plasmid pRTL1 of *Rhodococcus rhodochrous* NCIMB13064 (Poelarends et al., 2000b). An insertion sequence IS*2112* was located upstream of the *dhaA* gene (Kulakova et al., 1995; Kulakova et al., 1997; Kulakova et al., 1999; Poelarends et al., 2000a). Significant similarity was detected with insertion elements

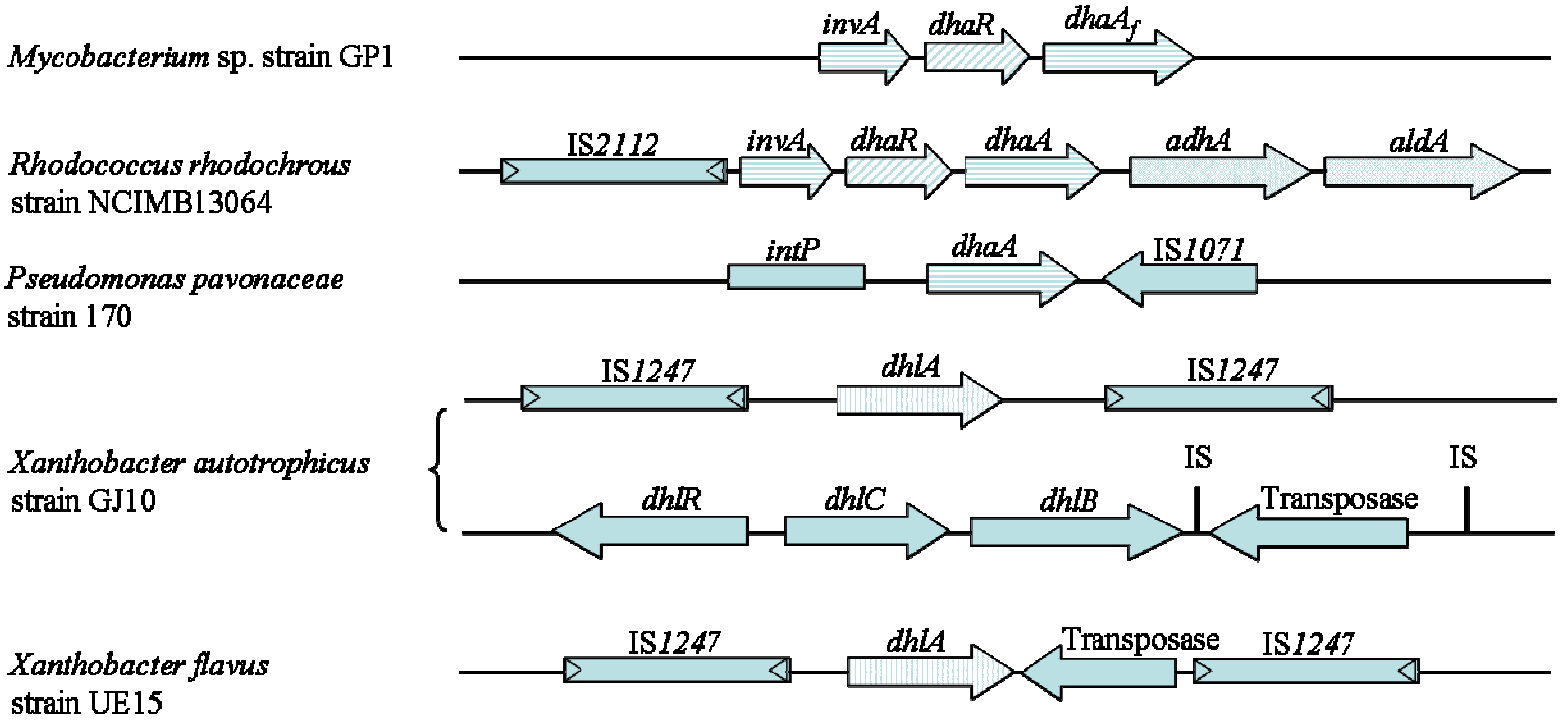

Figure 1.8 Organization of haloalkane dehalogenase genes of *Mycobacterium* sp. strain GP1, *Rhodococcus* sp. strain NCIMB13064, *Pseudomonas pavonaceae* 170, *Xanthobacter autotrophus* GJ10, and *Xanthobacter flavus* UE15. Blocks represent insertion sequences. *Mycobacterium* sp. strain GP1 contains the haloalkane degradative gene, *dhaAf* , the regulatory gene, *dhaR*, the putative invertase gene *invA* (Poelarends et al., 2000b). *Rhodococcus* sp. strain NCIMB13064 contains the degradative gene *dha*, *adhA* and *aldA*, the regulatory gene, *dhaR*, the putative invertase gene *invA* and insertion elements IS*2112* (Poelarends et al., 2000b). *Pseudomonas pavonaceae* 170 contains the *dhaA* , the putative invertase gene and insertion element IS*1071* (Poelarends et al., 2000a). *Xanthobacter autotrophus* GJ10 contains the dehalogenase gene, *dhlA* flanked by two copies of insertion sequence IS*1247*, this bacterium also contains a haloacids dehalogenase, *dhlB* and its regulator, *dhlR*, the trasporter gene, *dhlC*, and transposase beside it. *Xanthobacter flavus* UE15 contains *dhlA* adjacent to transposase and flanked by two copies of insertion sequence, IS*1247* (Song et al., 2004).

isolated from *Mycobacterium* species (Kulakova et al., 1999). The 1,3-dichloropropene-degrading bacterium *Pseudomonas pavonaceae* strain 170 (Verhagen et al., 1995), possessed a DNA segment including a *dhaA* gene and an insertion element IS*1071* located down stream of *dhaA* gene (Poelarends et al., 2000a).

The gene of 1,3-dichloropropene utilizing *P. pavonaceae* strain 170, *dhaA* was shown to be identical to the *dhaA* gene of *Rhodococcus rhodochrous* strain NCIMB13064 (Poelarends et al., 1998; Poelarends et al., 2000a). The 1,2-dibromoethane-degrading bacterium *Mycobacterium* sp. GP1 contained a DNA region which included a regulatory protein *dhaR* and $dhaA_f$ genes (Poelarends et al., 1999; Poelarends et al., 2000a; Poelarends et al., 2000b).

The *dhlA* gene of *Xanthobacter autotrophicus* GJ10 was cloned and sequenced (Keuning et al., 1985; Janssen et al., 1989), and showed *dhlA* flanked by two copies of insertion sequence IS*1247*, located on plasmid pXAU1 (Tardif et al., 1991). Two IS*1247* elements flanking *dhlA* in *X. flavus* strain UE15 were located on a plasmid, and were identical to the insertion elements of the GJ10 strain, indicating that this IS elements may be involved in *dhlA* gene transfer (Van der Ploeg et al., 1995; Song et al., 2004). The sequences of the transposase and the insertion sequences IS*1247* from strain UE15 were also reported to be identical to insertion element IS*2112* in *R. rhodochrous* strain NCIMB13064 (Kulakova et al., 1999; Song et al., 2004).

The *lin* genes for the γ-HCH degradation in *Sphingobium japonicum* UT26 are dispersed on three large replicons (Chr1, Chr2, and a plasmid PCHQ1), and are associated with IS*6100* insertion sequence (Nagata et al., 2007). Comparing strain UT26 with other Sphingomonads showed that *lin* genes are divided in to two types, one located on the DNA regions unique to strain UT26 (*linA* to *linF*), and the other located in conserved regions (*linGHIJ* and *linKLMN*) (Nagata et al., 2007).

The haloalkane dehalogenase gene (*dhaA*) in the 1-chlorobutane-degrading bacterium *R. rhodochrous* NCIMB13064 is regulated by the regulatory gene, *dhaR* (Figure 1.8) (Poelarends et al., 2000a). The 1,2-dibromoethane-degrading bacterium *Mycobacterium* sp. GP1 contained a DNA segment which included a regulatory protein *dhaR* and $dhaA_f$ genes also with the same direction of transcription

(Figure 1.8) (Poelarends et al., 1999; Poelarends et al., 2000a; Poelarends et al., 2000b).

1.5.2 The organization of αHA dehalogenase genes

Biodegradation of halogenated compounds such as αHAs require the expression of catabolic genes, and also require the expression of appropriate transport and regulatory genes. The occurrence of related genes in clusters or operons (genes transcribed as a unit) has been well documented for catabolic genes. Clustering facilitates co-regulation and co-transcription that allow the expression of genes with related functions. Figure 1.9 summarizes the organization of αHA dehalogenase genes and associated genes in well characterized αHA-degrading bacteria.

Pseudomonas putida strain PP3, has two active dehlogenase genes (*dehI* and *dehII*) in separate but adjacent gene clusters, and a silent dehalogenase (*dehI*^{o}) located in the *dehII* operon (Hill et al., 1999). *Pseudomonas putida* AJ1 has *hadD* and *hadL* genes, organized in the same direct of transcription (Barth et al., 1992), as *hadD* and *hadL* gene of *Rhizobium* sp. (Figure 1.9). *Rhizobium* sp. also contains a third dehalogenases gene *dehE* that appeared to have a putative regulator gene upstream that transcribed in the opposite direction (Allison et al., 1983; Stringfellow et al., 1997).

Bacterial genes encoding related function, such as catabolic genes and their associated regulatory and transport genes, are often clustered within the bacterial genome. Closely clustered genes may be co-transcribed as a unit (operon) (Lawrence, 2002), and dehalogenase genes are often clustered, even when genes are from different families.

Several αHA dehalogenase genes are closely associated with IS elements and /or are contained in transposable elements. The Tn*DEH*, is a composite transposon flanked by identical copies of IS*Ppu12* (Figure 1.9) capable of high frequency insertion into several different *Proteobacteria*, and was also associated with dehalogenase gene silencing in strain PP3 (Weightman et al., 2002). Another dehalogenase gene, *dehH2* genes of *Delftia acidovorans* strain B carried on a transposon designated Tn*Had1* (Figure 1.9), flanked by two copies of IS*1071*, Tn*Had1*, along with dehalogenase

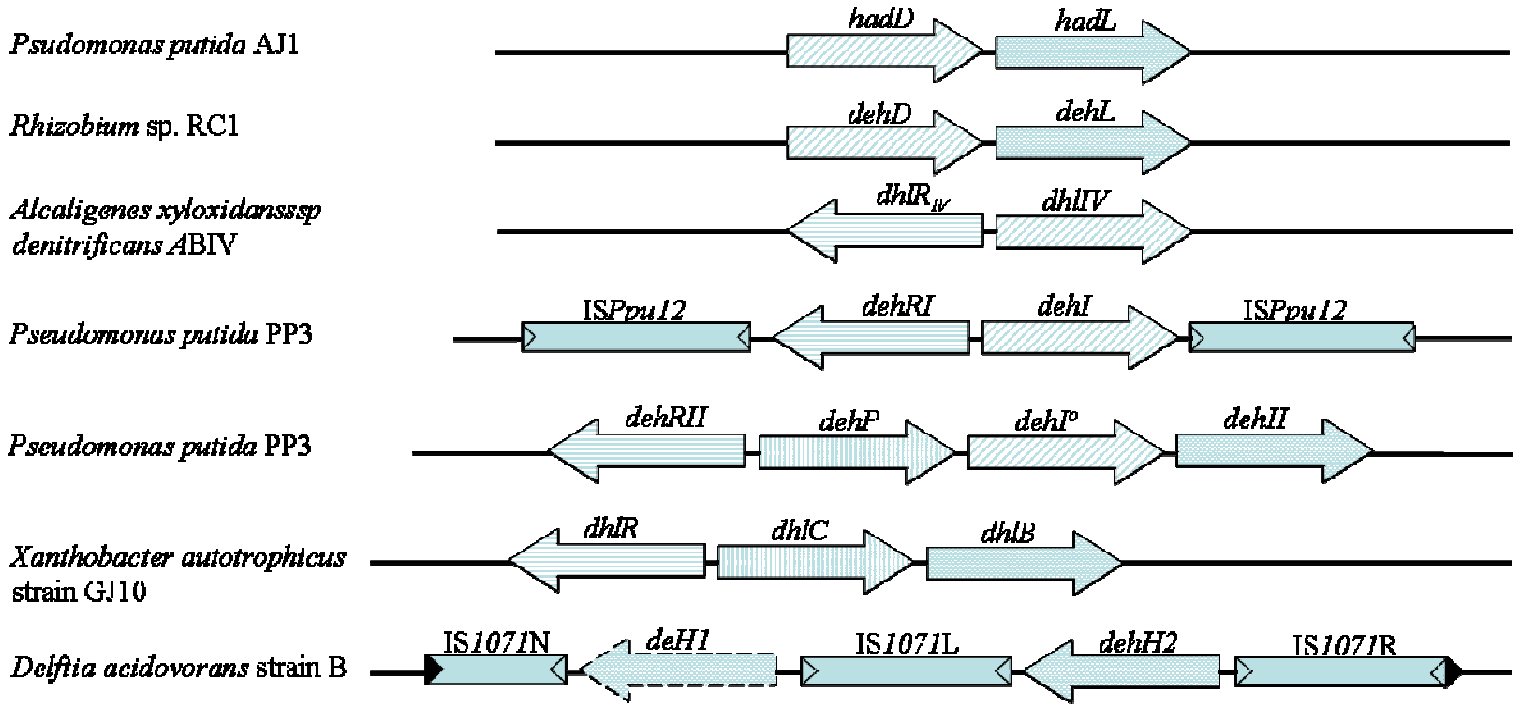

Figure 1.9 Structural organization of characterized αHA dehalogenase gene systems. The arrows represent the direction of the genes transcription. Group I and group II α-HA *deh* classified by Hill et al (1999) are represented by upward diagonal lined and dotted arrows, respectively. The open dotted arrow represents flouroacetate dehalogenase gene. Arrows with horizontal line patterns indicate putative σ^{54}-dependent regulator genes, and arrows with vertical line patterns indicate putative sodium dependent symport protein (permease) genes. Open rectangle with flanking unfilled triangles represent insertion sequence elements and inverted repeats, respectively. Filled triangles represent separate repeat sequences. *Pseudomonas putida* AJ1 and *Rhizobium* sp. contained *hadD* and *hadL*, *dehD* and *dehL*, respectively (Leigh et al., 1988; Barth et al., 1992). *Alkaligenes xyloxidans* ssp. *denitrificans* ABIV contained *dhlIV* and its regulator gene $dehlR_{IV}$ (Brokamp et al., 1996). *Pseudomonas putida* PP3 contained *dehI* and its regulator, $dehR_I$, flanked by two copies of insertion sequences, IS*Ppu12* within the Tn*DEH* (Thomas et al., 1992a; Thomas et al., 1992b; Topping et al., 1995; Weightman et al., 2002; Teuten and Reddy, 2007). *P. putida* PP3 also contain *dehII* and its regulator, $dehR_{II}$, a permease *dehP*, the silent $dehI^{o}$ within the *dehII* gene cluster (Dodds, 2003; Laturnus et al., 2005). *Xanthobacter autotrophicus* GJ10 contains regulator a *dhlR*, a premease *dhlC*, and *dhlB* (Van der Ploeg et al., 1991; Van der Ploeg et al., 1995). *Delftia acidovorans* strain B contained *dehH2* flanked by two copies of IS*1071* within Tn*Had1*, and *dehH1* adjacent to truncated insertion sequence, IS*1071*N within Tn*Had2* (Sota et al., 2002).

dehH1, are also contained within a second transposon designated Tn*Had2* (Figure 1.9), and both transposons are located on plasmid pUO1 (Sota et al., 2002). Dehalogenase gene from *Pseudomonas putida* strain 109, *dehH109*, is located on plasmid pUOH109 (Kawasaki et al., 1994), and *Alcaligenes xyloxidans* ssp. *denitrificans* ABIV contains a non-stereoselective dehalogenase gene *dhlIV*, located on plasmid pFL40.

1.5.2.1 Silent (cryptic) αHA dehalogenase genes

Silent or cryptic genes are not normally expressed, but they can be activated by mutation, recombination, insertion elements or other genetic mechanisms (Hall et al., 1983). Stress such as starvation (toxic compound) may influence genetic rearrangement and might be connected to the activation of silent dehalogenase genes and catabolic transposons (Fetzner and Lingens, 1994). *Burkholderia cepacia* MBA4 contains a silent dehalogenase gene, *chd1*, which was cloned and expressed to show Chdl was active towards L-isomer of 2MCPA, and had a long leader sequence typical of periplasmic enzymes (Tsang and Sam, 1999). *Pseudomonas putida* PP3 contain a silent *dehI*^{o} gene of *Pseudomonas putida* PP3, located upstream of permease gene, *dehP* within the *dehII* gene cluster (Hill et al., 1999; Dodds, 2003). *Agrobacterium tumefaciens* RS5 also produced the silent *dhlS5I* gene, was cloned and expressed in *E. coli* as L-α-HA specific dehalogenase (Schwarze et al., 1997; Köhler et al., 1998). The decryptification (switching on) of dehalogenase function has also been reported in a *Rhizobium* sp., capable of utilizing 22DCPA and 2MCPA (Leigh et al., 1986).

Several haloacid dehalogenase specific regulatory genes have been identified (Figure 1.9). Brokamp et al. (1997) reported the presence of regulatory gene *dehlR*$_{IV}$ in *Alkaligenes xyloxidans* ssp. *denitrificans* ABIV located upstream of *dhlIV*. Other regulatory genes include: *dehRI* associated with *dehI*, *dehRII* with *dehII* of *Pseudomonas putida* PP3 (Topping et al., 1995; Dodds, 2003); *delR* and *dhlB* of *Xanthobacter autotrophicus* GJ10 (Van der Ploeg et al., 1995); and a partial regulator-like sequence with *dehE* of *Rhizobium* sp. (Stringfellow et al., 1997). All of these regulator genes show nucleotide sequence similarity with σ^{54}-dependent activator protein genes.

1.6.1 Bioremediation and detoxification

Dehalogenase producing organisms are potentially useful in the bioremediation of terrestrial and aquatic environments contaminated with halogenated compounds either using *in situ* or *ex situ* methods (Chaudhry and Chapalamadugu, 1991; Hardman, 1991; Stroo, 1992; Fetzner and Lingens, 1994; Dravis et al., 2001). For example, *Xanthobacter autotrophicus* GJ10, containing haloalkane dehalogenase DhlA and αHA dehalogenase DhlB, has been used in bioremediation of ground water contaminated with 1,2-dichloroethane (Stucki and Thueer, 1995). The bioremediation potential of DhlB of *X. autotrophicus* GJ10 was also evaluated in the dechlorination of 1,2-dichloroethane using immobilized cells (Meusel and Rehm, 1993). DhaA, haloalkane dehalogenases from *R. rhodocochrous* NCIMB13064 was used to convert two gas-phase halogenated aliphatic hydrocarbons, 1-chlorobutane and 1,3-dichloropropane to their corresponding alcohols (Dravis et al., 2000). Dehalogenase genes, *dhlA* and *dhlB* of *X. autotrophicus* GJ10 were introduced to tobacco plants which were then able to degrade 1,2-dichloroethane (Mena-Benitez et al., 2008). This study gives insight into low cost phytoremediation of environment contaminated with halogenated compounds. Haloalkane dehalogenases also have been shown to have uses in combating chemical warfare as it can act against toxic agent like mustard gas (Nagata et al., 2005; Prokop et al., 2006). Greeg et al. (1998) transformed a rumen bacterium with the gene coding for *Deltia acidovorans* fluoroacetate dehalogenase and found that when the genetically modified bacterium was introduced to sheep, they showed reduced toxicological symptom to fluoroacetate.

1.6.2 Synthesis of chiral compounds

Dehalogenases can also be used as industrial biocatalysts to produce intermediates or in synthesis of optically active compounds (Motosugi et al., 1984; Fetzner and Lingens, 1994). They are useful as biocatalyst in converting organohalogens in stereospecific manner (Motosugi et al., 1984; Swanson, 1999). L-2MCPA, which is an intermediate in the synthesis of a phenoxypropionic acid herbicide, is obtained from racemic mixture of 2MCPA when D-2MCPA of the racemic mixture is selectively degraded with D-2-haloacid dehalogenase, HadD of *P. putida* strain AJ1 (Parker and Colby, 1995). DehCI (L-2-haloacid dehalogenase) from *Pseudomonas* sp.

strain CBS3 was also used for production of D-2MCPA and D-lactate from racemic 2MCPA (Ordaz et al., 2000). L-αHA specific H-109 of *Pseudomonas putida* 109 was also used in immobilization studies in biotransformation of racemic 2-bromopropionic acid (Vyazmensky and Geresh, 1998).

1.7 Aims

The aim of this study was to explore bacterial community and dehalogenase gene diversity and changes in diversity during degradation of pollutant, 2-monochloropropionic acid (2MCPA) in a standard OECD Ready Biodegradation Test (RBT) using combination of cultivation-dependent and cultivation-independent approaches.

Chapter 3 describes an investigation of 2MCPA RBT microbial community changes following separate inoculation with lyophilized activated sludge and soil. It was hypothesized that molecular genetic analysis of the bacterial community could be used to link community composition and its function. The effect of the different inoculum size of soil on the outcome of RBTs during 2MCPA degradation was also investigated. It was hypothesized that higher inoculum concentration would carry greater degradative ability for 2MCPA, resulting in quicker degradation of 2MCPA, whilst lower concentration would reduce degradative ability resulting in significant slower degradation of 2MCPA.

Chapter 4 describes the isolation of dominant bacterial strains from the RBTs able to utilize 2MCPA. It was hypothesised that bacterial community analysis using cultivation dependent methods would result in reducing the diversity of 2MCPA degraders and could result in the isolation of non representative strains from the RBTs. The preliminary characterization of isolated bacteria and their dehalogenases by molecular genetic analysis, enzyme assays, and gel zymography was also undertaken.

The experimental approach used in this study is summarized in Figure 1.10.

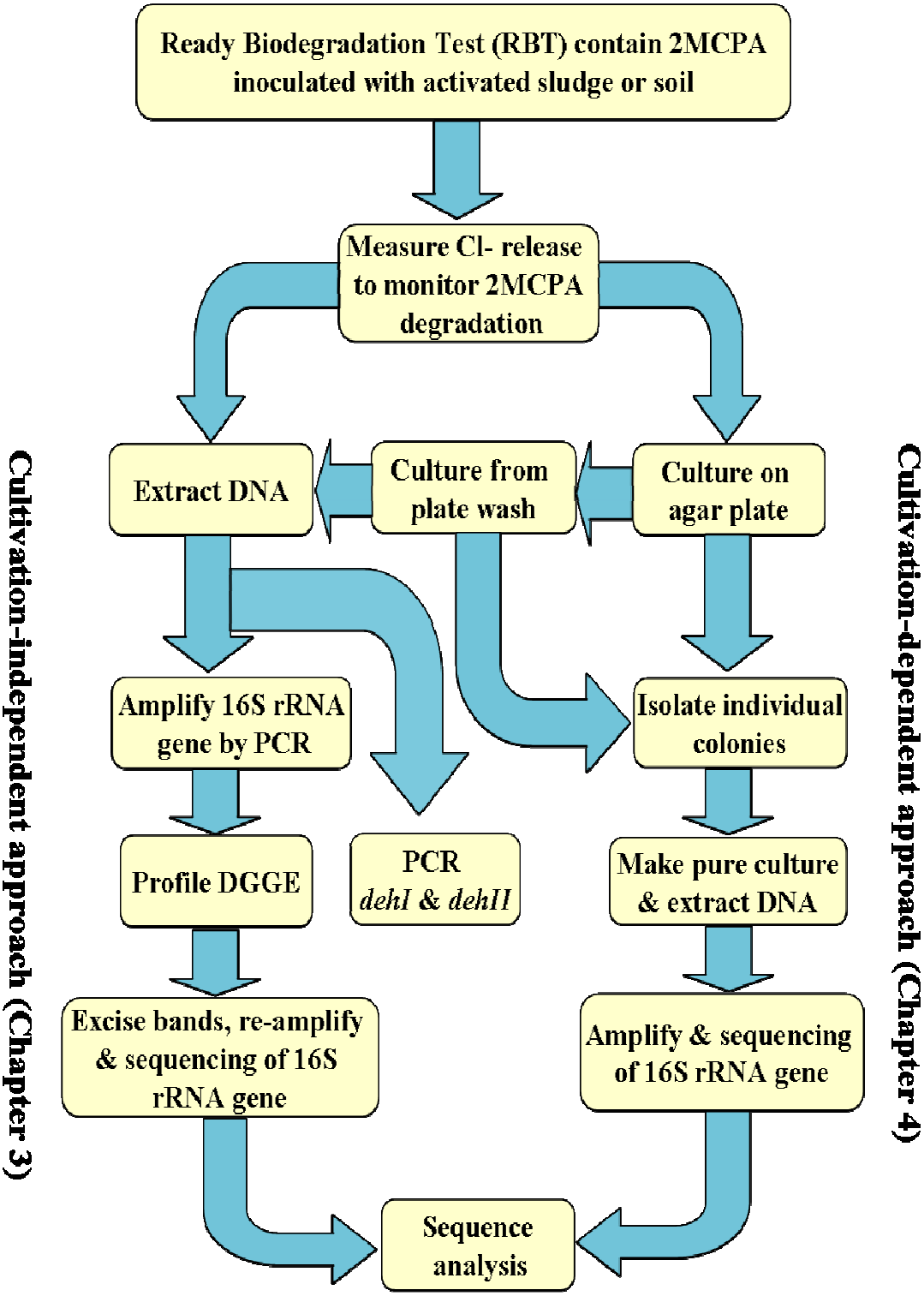

Figure 1.10 A diagram shows the experimental approaches used in this study

Chapter 2. Materials and Methods

2.1 Organisms and media

Enrichments and cultivation of the strains (*Herbaspirillum* sp. KH7, *Methylobacterium* sp. KH4, *Afipia* sp. KH3, and *Rhizobium* sp. KHD; Table 2.1), were done in standard basal salts minimal medium (SBS) (Slater et al., 1979), containing the following (g/l dH_2O): 1.5 K_2HPO_4, 0.5 KH_2PO_4, 0.5 $(NH4)_2SO_4$, and 0.2 $MgSO_4.7H_2O$. The medium was autoclaved and supplemented with filter sterilized trace elements solution (10 ml/l) and filter sterilized 2MCPA (2, 10 or 15 mM). Trace elements solution contained (g/l): 12.0 Na_2EDTA $2H_2O$; 2.0 NaOH; 1.0 $MgSO_4.7H_2O$; 0.4 $ZnSO_4.7H_2O$; 0.1 $CuSO_4.5H_2O$; 10.0 Na_2SO_4; 0.1 $Na_2MoO_4.2H_2O$; 2.0 $FeSO_4.7H_2O$; 0.4 $MnSO_4.4H_2O$; and 1.0 $CaCl_2$; in dH_2O.

All halogenated and other substrates were prepared as 1 M stock solutions, adjusted to pH 6.8, filter sterilised and stored at 4^oC in dark. Media and stock solutions were sterilized by autoclaving at 121^oC for 25 min (unless heat labile). Heat sensitive compounds, such as chlorinated carbon sources, trace elements solution, were sterilized by filtration using 0.2 μm pore diameter filters (Sartorius, UK).

Agar plates were prepared by adding purified agar (Difco, UK) to the SBS medium at a final concentration of 1.5%. The mixture was autoclaved at 121^oC for 15 min and cooled to 55^oC in a water bath. Filter sterilized carbon source 2MCPA was added aseptically to the molten agar, mixed thoroughly and poured into Petri dishes in a laminar flow cabinet and dried. Purity of the isolated strains was regularly checked by plating out serially diluted culture samples onto nutrient agar, SBS medium containing 2MCPA, R2A medium (Reasoner and Geldreich, 1985), and Yeast Extract Manitol agar (YEM) medium (Vincent, 1970), and nutrient agar media. Stock cultures of all strains were stored in DMSO 8% at -80^oC.

The primary aerobic enrichment carried out as Ready Biodegradation Test, RBT (see section 2.4) was done by liquid batch culture in a previously defined mineral medium (OECD, 1992). The medium (Sturm) was made as follows: 5 ml of solution A; 0.5 ml each of solutions B, C and D into a total volume of 500 ml. Solution (A) contained (in g/l): 8.50 KH_2PO_4; 21.75 K_2HPO_4; 33.40 $Na_2HPO_4.2H_2O$; and 0.50 NH_4Cl; solution (B) contained (in g/l): 36.40 $CaCl_2.2H_2O$; solution (C) contained (in g/l): 22.50 $MgSO_4.7H_2O$; and solution (D) contained (in g/l): 0.25 $FeCl_3.6H_2O$, in

deionised water (dH_2O). Filter sterilized 2MCPA was added as sole carbon and energy sources at a final concentration of 0.55 mM (= 20 mg carbon/l) for the RBTs.

Table 2.1 Bacterial strains used in this study

Strain	Source of isolate reference
Pseudomonas putida PP3*	(Senior et al., 1976; Weightman et al., 1979)
Herbaspirillum sp. KH17	Soil, this study (Chapter 4)
Methylobacterium sp. KH4	Soil, this study (Chapter 4)
Afipia sp. KH3	Activated sludge, this study (Chapter 4)
Rhizobium sp. KH31	Activated sludge, this study (Chapter 4)

* Strain PP3 was originally isolated from a microbial community obtained by continuous-flow enrichment with 22DCPA as the sole carbon and energy source, derived from parental strain PP1 which expressed no dehalogenase, mutation event occurred led to the switching-on of regulated *dehI, dehII* expression (Senior et al., 1976).

2.2 Growth rate estimation

To determine the growth rate of the isolated strains, pure cultures which were stored at -80^{o}C were used to inoculate 10 ml of minimal media contained 2MCPA at final concentration of 10 mM, and from this when 80% dechlorination was achieved, 1 ml was used to inoculate 100 ml minimal medium containing 2MCPA at a final concentration of 10 mM. Samples were removed periodically and growth rate was determined by measuring optical density of the cultures at 600 nm using JENWAY 6300 spectrophotometer and biodegradation was measured through chloride release (mM) (see section 2.10) in liquid cultures over time (Weightman and Slater, 1980; Slater et al., 1997).

Bacteria were grown aerobically at 20^{o}C/170 rpm. For higher accuracy, experiments were done in triplicate. Growth rates were determined using the following calculations: $\mu = (\ln Nt - \ln No)/T$, where Nt represents natural logarithm of optical density (cell/ml) measured, or halide (mM) released at the time t and No is the absorbance or chloride released at t_o. Doubling time was calculated according to the equation: $Td = \ln 2/\mu$. Controls lacking the bacterium were included to detect spontaneous release of chloride ions.

2.2.1 Substrate ranges of the isolates

The ability of the isolates to utilize other halogenated compounds and carbon sources was determined by growth in liquid culture on SBS supplemented with 5 mM of the following substrates: glucose; sodium acetate; sodium succinate; monochloroacetate (MCA); dichloroacetate (DCA); trichloroacetate (TCA); dichloropropionate (DCPA); monochlorobutrate (2MCBA). Growth was estimated based on Cl^- release after aerobic incubation at 20^{o}C.

2.3 Sources of samples used to inoculate RBTs

Freeze dried activated sludge (freshly collected activated sludge on 31 January 2007) was sampled from Coslech waste water treatment plant (WWTP) – situated near Cardiff at 51°30'7.00"N and 3°20'37.16"W – which treats municipal waste water with some industrial waste water input. Sludge sample was centrifuged at 20,000 xg for 20 min at 20^{o}C, the supernatant was removed and discarded, leaving 5-10 ml with which to resuspend the pelleted sample. Re-suspended sludge pellets were dispensed as 1 ml aliquots, snap frozen in an acetone–dry ice bath and lyophilized under vacuum and were stored at -20^{o}C.

Soil samples were collected from the front lawn of Main Building, Cardiff University (November 2007) at 51°29'15.07"N; 3°10'46.94"W. Soil was used directly for RBT enrichments as described below.

2.4 Ready biodegradation test (RBT)

The RBT enrichment cultures (OECD, 1992) were inoculated by addition of freeze-dried activated sludge (0.02% w/v), or soil (0.10% and 0.05% w/v) to 500 ml sterile mineral media in 1 l conical flasks containing 0.55 mM 2MCPA as the sole carbon and energy source. Chloride concentration of 1 mM was achieved by adding sterile NaCl to the RBTs. The RBTs were incubated at 20^{o}C with shaking at 170 rpm. Samples (in triplicate, see section 2.10) were removed aseptically for Cl^- analysis, DNA extraction (stored -20^{o}C until use), PCR (see sections 2.5 and 2.6), and cultivation (following serial dilution). Time zero samples were taken immediately after inoculation, followed by further daily samples (Figure 2.1).

2.4.1 Preparation of liquid sub-cultures and isolation of strains from the RBTs

Samples (200 µl) were taken from the RBTs following serial dilution (10^{-1}-10^{-7}), and used to inoculate solid SBS medium containing 2MCPA (15 mM). For activated sludge RBT (AS-RBT), three plates represented day 11, day 16, and day 20 (named S11, S16, S20) were selected for biomass (plate wash) harvesting and the biomass was used to inoculate fresh liquid sub-cultures SBS medium containing 2 mM and 10 mM 2MCPA. Following dechlorination in the sub-cultures (50% and 100%), samples were used to, inoculate fresh SBS 2MCPA medium (2 mM and 10 mM), this procedure continued until third sub-culture was obtained, following which pure cultures were isolated from the liquid sub-cultures on plates. To isolate 2MCPA-degrading bacteria from soil RBTs the same procedure was used, but without extensive liquid sub-culturing and DGGE profiling, and only at 10 mM 2MCPA.

The cultures obtained were purified by streaking single colonies onto SBS plates containing 2MCPA, and cultures were further purified by picking and streaking single colonies onto fresh medium and checking ability to dechlorinate in SBS liquid medium containing 2MCPA as sole source of carbon and energy. After complete dechlorination (100%), purity of the cultures was checked again by cultivation on nutrient agar and SBS 2MCPA plates. 16S rRNA genes from purified cultures were sequenced directly following PCR with appropriate primers (see section 2.6.1).

2.5 Extraction of DNA

Total nucleic acid (DNA) from RBTs (3 ml) or pure cultures (1 ml) was obtained by using MP Biomedicals FastDNA®SPIN Kit for soil according to the manufacturer's instructions. Briefly, cells lysed by bead beating method in lysing matrix E, cell debris and lysing matrix were removed by centrifugation, and DNA was purified from the supernatant using silica based spin filters, and then eluted with DEPC treated H_2O.

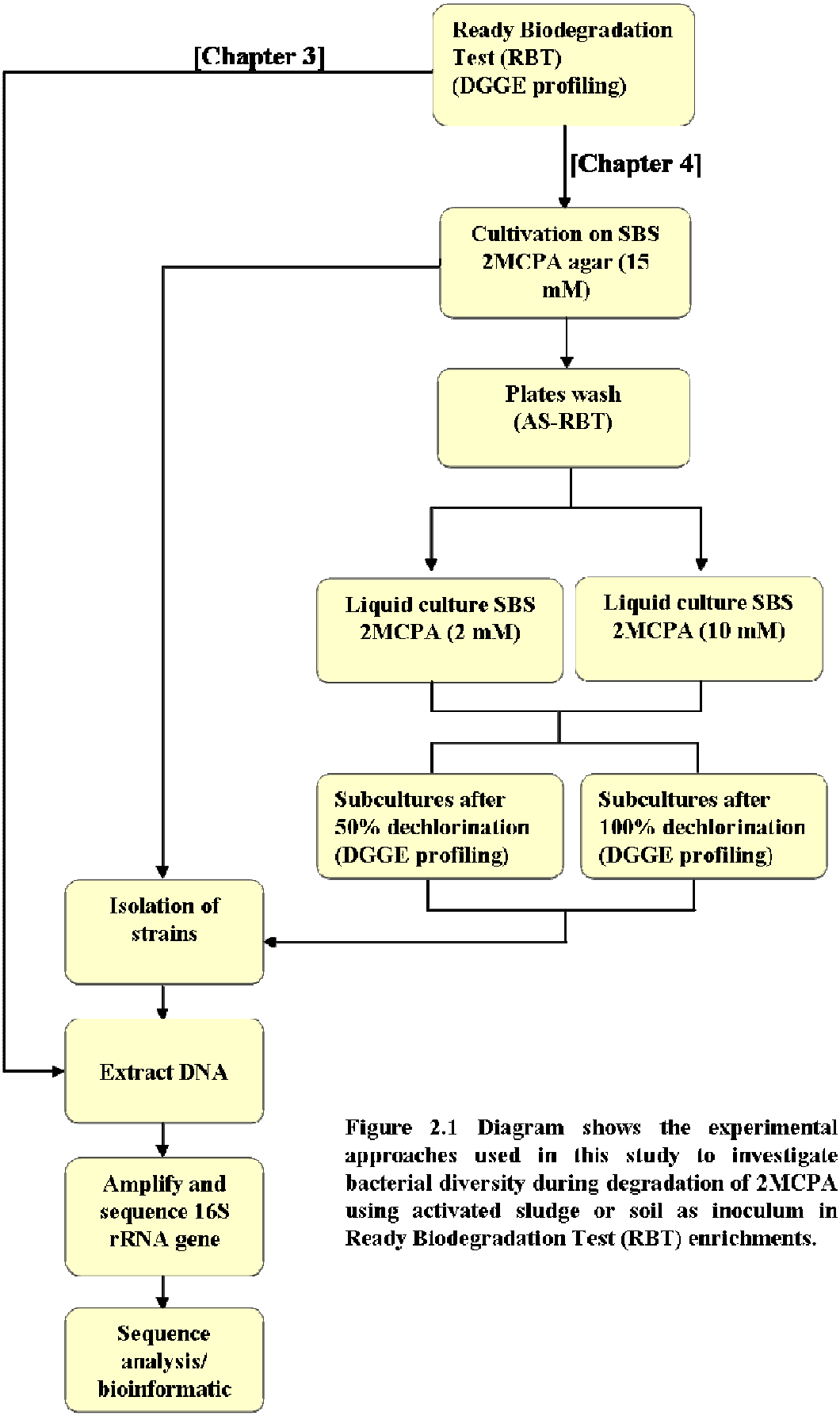

Figure 2.1 Diagram shows the experimental approaches used in this study to investigate bacterial diversity during degradation of 2MCPA using activated sludge or soil as inoculum in Ready Biodegradation Test (RBT) enrichments.

DNA samples were stored at -20^{o}C until required. This method has been shown previously to be a rapid and efficient method for extracting high quality DNA from complex environmental samples (Webster et al., 2003; Newberry et al., 2004).

2.6 PCR amplifications of target genes

All PCR reactions were carried out using a DNA engine Dyad^{TM} thermal cycler gradient block (MJ Research). Primer sequences used in this study are listed in Table 2.2.

2.6.1 PCR amplification of 16S rRNA gene

The amplification of partial 16S rRNA gene sequences was performed using the primer pairs 357FGC and 518R (Muyzer et al., 1993); or 27F and 1492R (Lane, 1991) (Table 2.2). The PCR mixture (50 µl) contained: 1x GoTaq® Flex Buffer (Promega); 1.5 mM $MgCl_2$ (Promega); 0.25 mM dNTPs (Promega); 0.2 µM of both oligonucleotide primers; 1.25 U *Taq* DNA polymerase (Promega) and 1 µl of template DNA, made up to volume of 50 µl with DEPC treated RNase free water (Severn Biotech). The following PCR programme was used for amplification using 357F-GC and 518R: an initial 5 min denaturation step at 95^{o}C was followed by 10 cycles of three steps PCR programme (94^{o}C for 30 seconds, 55^{o}C for 30 seconds, and 72^{o}C for 1 min), this was followed by another 25 cycles of three steps (92^{o}C for 30 seconds, 52^{o}C for 30 seconds, and 72^{o}C for 1 min) and a final 10 min at 72^{o}C. Conditions used for PCR amplification using 27F, 1492R were 95^{o}C for 2 min; 36 cycles of three steps (94^{o}C for 30 seconds, 52^{o}C for 30 seconds, and 72^{o}C for 1.5 min) and a final 5 min at 72^{o}C.

2.6.2 PCR amplification of αHA dehalogenase (*deh*) genes

Genomic DNA from *Pseudomonas putida* strain PP3, provided by Dr. Louise O'Sullivan, was used as a control for PCR amplification of group I and group II *deh* genes of αHA. The amplification of αHA dehalogenase genes (*dehI, dehII)* was performed by using degenerate primers described by Hill et al. (1999) (Table 2.2), *dehI* (*dehI* short ca. 230 bp, $dehI_{for1}$ and $dehI_{rev1}$; *dehI* long ca. 504 bp, $dehI_{for1}$, $dehI_{rev2}$), and *dehII* (ca. 422 bp). The *dehI* gene amplification were performed in a PCR mixture (50 µl) contained 1x GoTaq® Flex Buffer (Promega); 1.5 mM $MgCl_2$

(Promega); 0.2 mM dNTPs (Promega); 4 μM $dehI_{for1}$ and $dehI_{rev1}$/$dehI_{rev2}$; 1 U *Taq* DNA polymerase (Promega) and 1 μl of template DNA, made up to volume of 50 μl with DEPC treated RNase free water (Severn Biotech Ltd). The following PCR cycle was used for *dehI*: 94°C for 2 min; 20 cycles of 92°C for 20 seconds, 70°C for 30 seconds (-1°C per cycle), 75°C for 30 seconds; 20 cycles of 92°C for 20 seconds, 51°C for 30 seconds, 75°C for 30 seconds and a final extension step of 75°C for 5 min.

PCR amplification of *dehII* was performed as for *dehI*, using 6 μM $dehII_{for1}$ and $dehII_{rev1}$ primers. The PCR programme for *dehII* was: 94°C for 10 min; 36 cycles of 94°C for 45 seconds, 55°C for 2 min, 75°C for 45 seconds and a final extension step of 75°C for 5 min.

PCR to amplify vector inserts were done with M13F & M13R primer pairs (Table 2.2) PCR mixture (50 μl) contained 50 pmol of each primer, 0.25 U of *Taq* polymerase, 500 μM dNTPs, 3 mM $MgCl_2$, 5x buffer containing 50 mM KCl and 10 mM Tris-HCl, pH 9.0, using the following PCR cycle: 94°C for 10 min, 30 cycles of 92°C for 45 seconds, 50°C for 45 seconds, 75°C for 1 min and a final extension step of 75°C for 5 min.

2.6.3 PCR amplification of DNA gyrase subunit B (*gyrB*) gene

The *gyrB* gene was amplified using the primers UP-1 and UP-2r (Table 2.2), and PCR described by Yamamoto and Harayama (1995). The PCR reaction (50 μl) contained 1 x GoTaq® Flex Buffer (Promega); 1.5 mM $MgCl_2$ (Promega); 0.2 mM dNTPs, 1 μM of UP-1 and UP-2r primer, 2.5 U *Taq* DNA polymerase and 1 μl of template DNA. The following PCR condition was used: 94°C for 5 min; a total of 30 cycles of denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, and primer extension at 72°C for 2 min; 72°C for 5 min. The *gyrB* PCR products were purified using Montage PCR Centrifugal Filter Devices (Millipore), according to the manufacturer's instructions. Two specifically designed primers, UP-1S and UP-2Sr were used for sequencing the *gyrB* PCR products.

Table 2.2 Primers used in this study for PCR amplification

Primers	Target gene	Primer sequence (5'- 3')[a]	Reference
357F[b,c,d]	16S rRNA gene	CCTACGGGAGGCAGCAG	(Muyzer et al., 1993)
518R[e]	16S rRNA gene	ATTACCGCGGCTGCTGG	(Muyzer et al., 1993)
27F[f]	16S rRNA gene	AGAGTTTGATCMTGGCTCAG	(Lane, 1991)
1492R[f]	16S rRNA gene	GGTTACCTTGTTACGACTT	(Lane, 1991)
UP-1	*gyrB*	GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNAARTTYGA	(Yamamoto and Harayama, 1995)
UP-2r	*gyrB*	AGCAGGGTACGGATGTGCGAGCCRTCNACRTCNGCRTCNGTCAT	(Yamamoto and Harayama, 1995)
UP-1S		GAAGTCATCATGACCGTTCTGCA	(Yamamoto and Harayama, 1995)
UP-2Sr		AGCAGGGTACGGATGTGCGAGCC	(Yamamoto and Harayama, 1995)
$dehI_{for1}$	*dehI*	ACGYTNSGSGTGCCNTGGGT	(Hill et al., 1999)
$dehI_{rev1}$	*dehI*	AWCARRTAYTTYGGATTRCCRTA	(Hill et al., 1999)
$dehI_{rev2}$	*dehI*	SGCMAKSRCNYKGWARTCACT	(Hill et al., 1999)
$dehII_{for\ 1}$	*dehII*	TGGCGVCARMRDCARCTBGARTA	(Hill et al., 1999)
$dehII_{rev1}$	*dehII*	TCSMADSBRTTBGASGANACRAA	(Hill et al., 1999)
M13F		GTAAAACGACGGCCAGT	(O'Sullivan et al., 2008)
M13R		CAGGAAACAGCTATGAC	(O'Sullivan et al., 2008)
270		TGCGCGCGGG	(Mahenthiralingam et al., 1996)
272		AGCGGGCCAA	(Mahenthiralingam et al., 1996)
277		AGGAAGGTGC	(Mahenthiralingam et al., 1996)

a. Y = C or T; N = A, T, G or C; S = C or G; M = A or C; K = G or T; R = A or G; W = A or T; V = A, C or G; D = A, G or T; B = C, G or T. All primers were synthesised by MWG Biotech.

b. Primer numbers designated according to 3'binding position with reference to *E. coli* 16S rRNA nucleotide numbering (Brosius et al., 1981).

c. For DGGE, this primer has an additional 5' GC clamp: CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG (Muyzer et al., 1993) and is referred to as 357FGC in this report.

d. For re-amplification of excised DGGE bands, this primer had a 5' GC linker, which also contains M13R site: CAGGAAACAGCTATGACGGGCGGGGCGGGGGCACGGGGGG (O'Sullivan et al., 2008) and is referred to as 357F-GC-M13R

e. For re-amplification of excised DGGE bands, this primer had a 5' AT linker region, which also contains M13F site: GTAAAACGACGGCCAGTAAATAAAATAAAAATGTAAAAAAA (O'Sullivan et al., 2008) and is referred to as 518R-AT-M13F

f. Primers, 16S- 27F and 16S-1492R (Lane, 1991) were used to amplify the 16S rRNA gene of the pure isolates

2.6.4 Random amplified polymorphic DNA (RAPD) analysis

PCR-RAPD was carried out using RAPD primers 270 (5'-TGCGCGCGGG-3'), 272 (5'-AGCGGGCCAA) and 277 (5'-AGGAAGGTGC) and PCR described by Mahenthiralingam et al. (1996) (Table 2.2). The PCR reaction (50 µl) contained: 1 x GoTaq® Flex Buffer (Promega); 3 mM $MgCl_2$ (Promega); 0.25 mM dNTPs, 1.6 µM of 270 or 272 or 277 primer, 1.25 U of *Taq* DNA polymerase and 4 µl template DNA. The following conditions were used for PCR amplification: 4 cycle of 94^{o}C for 5 min, 36^{o}C for 5 min, 72^{o}C for 5 min; 30 cycles of 94^{o}C for 1 min, 36^{o}C for 1 min, 72^{o}C for 2 min and a final extension step of 72^{o}C for 10 min.

2.7 Agarose gel electrophoresis

All the PCR products and extracted DNA were examined by electrophoresis in agarose gel (Sambrook et al., 1989) and quantified with Hyper ladder I (Bioline). Agarose gels were prepared with agarose concentration of 1.2 % (w/v) in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.3 – Severn Biotech Ltd.). PCR products mixed with loading dye (bromophenol blue) were loaded into the wells, and the gels were run at 100 V for 30 min. The gels were stained with SYBR safe™ DNA gel stain (Invitrogen) which was incorporated into the gel and electrophoresis buffer. Gels then visualized directly upon illumination with UV light and photographed using the Gene Genius Bio Imaging System (Syngene). RAPD-PCR products were loaded on 1.5% w/v agarose gel in 1 x TAE buffer and run at 100 V for 40 min, gel stained in ethidium bromide (0.5 µg/ml) for 30 min. Gels images were obtained as described above.

2.8 Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed with a Bio-Rad DCode™ system, Universal Mutation Detection System. PCR products amplified with primer 357FGC and 518R, were loaded onto a polyacrylamide gels (8% w/v) containing a denaturant gradient between 30% and 60% (100% denaturant being 7 M urea and 40% w/v formamide). Gels were poured using 50 ml volume Gradient Mixer (Fisher Scientific, UK). PCR products were loaded into the wells, gel electrophoresis was carried out in 1 x TAE buffer at 80 V for 10 min, and then at 200 V for about 5 h at 60^{o}C (Muyzer et al., 1993; Webster et

al., 2006). Polyacrylamide gels were stained with SYBR®Gold nucleic acid gel stain (Introgen) in 1 x TAE for 25 min at room temperature, and viewed under UV illumination. Gel images were captured with a Gene Genius Bio Imaging System (Syngene).

DGGE bands were excited from polyacrylamide gels using a scalpel and washed with DEPC treated RNase free water (Severn Biotech Ltd) for 10 min, then air dried for 10 min. Dried bands were crushed with sterile tips and re-suspended in 15 µl DEPC treated water and stored at -20^{o}C until required. The eluted DNA (1µl) was used as a template for re-amplified by PCR using 357F-GC-M13R and 518R-AT-M13F primers (O'Sullivan et al., 2008) using PCR conditions described for 16S rRNA gene (section 2.6.1; Table 2.2). Re-amplified PCR products were sequenced using either M13F or M13R.

PCR products of bands of some DGGE fragments that gave a poor sequence were re-amplified with same primers sets (357F-518R), and re-run on DGGE gel to determine DGGE band purity.

2.9 DNA sequencing and phylogenetic analysis

16S rRNA gene sequence analysis was carried out following PCR-DGGE as described in sections 2.6.1 and 2.8, except PCR products of 16S rRNA gene of the bacterial isolates which were amplified with 27F and 1492R primers, were sequenced directly.

Sequencing was performed using the ABI PRISM BigDye Terminator v3.1 cycle sequencing kit and run on an ABI 3130x1 Genetic Analyzer. 16S rRNA gene sequences were viewed using Chromas lite V2.01 (Technelysium Pty Ltd), then manually edited with the BioEdit program (Hall, 1999). Primer regions were removed from all 16S rRNA gene sequences prior to alignment. The amplified 16S rRNA gene sequences were compared with NCBI GenBank database sequences (http://www.ncbi.nlm.nih.gov) using the basic local alignment search tool (BLAST) to identify sequences with highest similarity (Altschul et al., 1997). The sequences were also compared with the closest matches found in the Ribosomal Database Project RDPII (www.cme.msu.edu/RDP/html/analyses.html) to know the closest match with

sequences of cultivated relatives in the RDP database (Cole et al., 2005). Sequences were aligned with references from GenBank using ClustalW2 (Larkin et al., 2007). Phylogenetic trees were constructed using nucleotide sequences in Mega v4.0 (Tamura et al., 2007) by the Neighbor-Joining method (Jukes and Cantor, 1969) with the Molecular Evolutionary Genetics Analysis package (MEGA version 4).

Nucleotide BLAST (Altschul et al., 1990) were used to find dehalogenase gene sequence homologues in GenBank database at the National Centre for Biotechnological Information. Clustal W2 (Larkin et al., 2007) was used for dehalogenase sequence alignment. BioEdit (Hall, 1999) software was used for manual sequence alignment editing. EditPlus was used for manual removal of primer sequences. Mega 4 software was used for construction of phylogenetic tree, using Neighbour-Joining method (Jukes and Cantor, 1969). All reference nucleotide sequences were obtained via EMBL database at the National Centre for Biotechnological Information with the following accession numbers: *dehI*$_{PP3}$ (AY138113), *dehI*$_{18a}$ (AJ1334458), *dehE* (Y15517), *dehI*$_{DA1}$ (AJ133455), *DL-dex* (U97030), *dehI* (HQ130469), *dehI* (FJ417108), *dehI* (HQ130465), *dehI*$_{DA2}$ (AJ133456), *dehI* (HQ130466), *dehI*$_{K55}$ (AJ133459), *dehI*$^{o}{}_{17a}$ (AJ133457), *dehI*$^{o}{}_{PP3}$ (AJ133461), *hadD* (M81841), and *dehD* (X93597).

2.10 Determination of chloride

The concentration of free chloride in the samples from RBT systems (section 2.4), sub-culture enrichments (section 2.4.1) and dehalogenase assays (section 2.12) was determined using Sherwood chloride analyzed model 926 (Sherwood scientific Ltd.) according to the manufacturer's instructions (Slater et al., 1985). 500 µl of samples were added to a gradient beaker (Sherwood scientific Ltd.) containing the following: 25 ml counting solution (10% (v/v) glacial acetic acid, 0.8% (v/v) concentrated nitric acid and 0.5 mM NaCl) and 100 µl indicator solution (6.0 g/l gelatine, 0.1 g/l thymol blue and 0.1 g/l thymol).

Free chloride ions titrated against Ag^{+} generated colourimetrically. The titration occurs by passing a constant current between two silver electrodes which provide a constant generation of silver ions (Ag^{+}) which combine with the chloride in the sample and form silver chloride (AgCl). When the chloride has been precipitated as

silver chloride, free silver ions begin to appear and the solution conductivity changes. This change is detected by the sensing electrodes and the read out will stop, displaying the results directly in milligrams of chloride per litre (mg/l) or milligrams % salts (mg %). The machine was calibrated using a standard 5.63 mM NaCl solution (200 mg Cl^-/l).

2.11 Preparation of cell-free extracts (CFEs)

For the determination of dehalogenase activity, cultures were harvested and cell-free extracts (CFE) were prepared as described by Thomas et al. (1992b). Strains were grown in 1 l conical flasks containing 500 ml of SBS medium contained 13.8 mM 2MCPA (0.5 g carbon /l), 20.8 mM Na-acetate (0.5 g carbon /l) and yeast extract (0.01%) and incubated at 20^{o}C with shaking at 170 rpm until 80-90% dechlorination was achieved. The purity of the cultures was regularly checked by plating on SBS agar containing 10 mM 2MCPA. Cells used to prepare cell-free extracts (CFEs) were harvested in late-exponential phase of growth (80-90% dechlorination) by centrifugation at 8000 xg for 10 min at 4^{o}C, washed twice in pre-cooled 50 mM Tris-H_2SO_4 buffer (pH 7), and re-suspended in 10-15 ml of the same buffer containing 2 mM dithiothreitol (DTT); added to stabilize dehalogenase activity. Cells were disrupted by two passages through pre-cooled (4^{o}C) French pressure cell at 1260 psi. After disruption of the cell suspention, cell debris (pellet) was removed by centrifugation at 20000 xg for 30 min at 4^{o}C. The resulting supernatant, cell-free extract (CFE) was kept on ice and used for dehalogenase activity assays, or aliquoted and stored at -80^{o}C until needed. The pellet was re-suspended in 5 ml 50 mM Tris-H_2SO_4 buffer (pH 7) with addition of 2 mM dithiothreitol (DTT) and stored at -80^{o}C until needed.

2.12 Dehalogenase assays

Dehalogenase assays were performed in glass test tubes at 30^{o}C in a total volume of 5 ml according to the method of Thomas et al. (1992b). 250 µl CFE was added to 200 mM Tris-H_2SO_4 buffer (pH 8) containing 1 mM NaCl. Before initiation of the reaction the reaction mixture was incubated without substrate at 30^{o}C in a water bath for 5 min, and the reaction was initiated by the addition of substrate to a final

concentration of 50 mM. Samples were removed from the mixture and assayed for Cl^- liberation at regular intervals (~ 5 min) over 30-40 min period, as described in section 2.8. Dehalogenation activity was routinely estimated with the following chlorinated substrates: MCA, DCA, 2MCPA, 22DCPA and 2MCBA. Protein concentration in the CFE was determined using Bradford reagent (Sigma) according to the manufacturer's instruction, using bovine serum albumin (BSA) as standard protein for calibration of the assay. Absorbance was measured at 595 nm where absorbance is proportional to the protein concentration (Bradford, 1976). One unit of dehalogenase activity was defined as amount of enzyme required to catalyze the conversion 1 μmol of substrate per min. Enzyme specific activities are given as μmol substrate converted /mg protein /min.

2.13 Native polyacrylamide gel electrophoresis (PAGE)

To separate different dehalogenases in the crude cell-free extracts electrophoretically, PAGE was performed using the DCode^{TM} universal mutation system (Biorad laboratories) according to the method of Weightman & Slater (1980). The running gel contained 375 mM Tris-H_2SO_4 buffer (pH 8.8), 8% acrylamide (consisting of an acrylamide: bis-acrylamide ratio of 37.5:1), 1 mM dithiothreitol (DTT), 0.1% ammonium persulphate and 0.075% tetramethylethylene diamine (TEMED). The stacking gel contained 123 mM Tris-H_2SO_4 buffer (pH 6.8), 3% acrylamide (consisting of an acrylamide: bis-acrylamide ratio 37.5:1), 0.1% ammonium persulphate and 0.05% TEMED. The running buffer (pH 8.3) contained 25 mM Tris, 192 mM glycine and 1 mM dithiothreitol (DTT). Gels were stored overnight at 4^oC. Cell-free extract (50-200 μl), prepared as described in section 2.11, was mixed with bromophenol blue (as marker) and glycerol at a ratio of 5:0.5:1, respectively. Samples (100 μl) were loaded into wells in the stacking gel, and electrophoresis was carried out at 200 V for 6-9 h at 4^oC (until the dye front reached nearly the bottom of the gel).

After electrophoresis, the gel was stained for dehalogenase activity, as described by Weightman and Slater (1980). The gel was incubated in 0.2 M Tris-H_2SO_4 buffer (pH 7.9) containing 50 mM of halogenated substrate (MCA or 2MCPA), at 30^oC for 30 min. Gels were then quickly washed twice in deionised water and immersed in 100 mM silver nitrate solution ($AgNO_3$). This resulted in precipitation of bands of AgCl

where free chloride ions were present in the gels, corresponding to regions of dehalogenase activity. The gel was then washed with deionised water to remove the excess $AgNO_3$ and was fixed by washing in 5% (v/v) acetic acid before being photographed.

2.14 Dehalogenase gene cloning

PCR purified products produced by $dehI_{for1}$ and $dehI_{rev1}$ (230 bp fragment of *dehI* gene) were used for cloning. Cloning was done using the pGEM-T easy vector system (Promega) and *Escherichia coli* JM109 (Yanisch-Perron et al., 1985) competent cells (Promega) according to the manufacturer's instructions with modifications. 50 ng pGEM®-T Easy vector (Promega, Southampton, United Kingdom), 0.2 U T4 DNA ligase and 5 µl 2x Rapid Ligation buffer were used for ligation protocol. Promega Rapid Ligation buffer includes following substrates: 300 mM Tris-HCl pH 7.8, 100 mM $MgCl_2$, 100 mM DTT and 10 mM ATP. Vector to PCR product ratios were adjusted to 3:1, 1:1, and 1:3 for optimised results. Ligation reaction was incubated overnight at 4^{o}C. Transformation was achieved by mixing 50 µl competent cells with 3 µl of ligation reaction, and cooling on ice for 20 min, before heat shocking cells for 45 seconds at 42^{o}C, then returning immediately to ice to a further 2 min. Cells were revived in 950 µl SOC (used in recovery step of *E.coli*, transformation efficiency) (0.5% w/v yeast extract, 2% w/v tryptone, 10 mM NaCl, 2.5 mM Kcl, 10 mm $Mgcl_2$, $MgSO_4$, 20 mM glucoe) medium (Introgen) at 37^{o}C with shaking at 150 rpm for 1.5 h, and 100-150 µl of the cell suspension was then plated out onto LB agar plates supplemented with ampicillin (100 µg/ml), IPTG (0.5 mM) and X-gal (80 µg/ml) and incubated at 37^{o}C for 24 h for selection of transformants.

Blue-white selection is achieved by process termed alpha complementation (Moosmann and Rusconi, 1996). The *lacZa* gene, present on the pGEM-T Easy vector, represents only a portion of the functional beta-galactosidase gene termed the alpha peptide. Only when fused with the omega peptide, encoded by a chromosomally located gene, *lacZΩ* , can a functional beta-galactosidase enzyme be produced, allowing the formation of blue colonies. If the *lacZa* gene disrupted following the ligation of nucleic acid fragment into the multiple cloning sites (MCS), alpha complementation of lacZΩ can not be achieved, and therefore no beta-galactosidase

will be formed, thus colonies will be white. Transformants were screened for cloned inserts by PCR (see section 2.6.2) and agarose gel electrophoresis (see section 2.7). Insert containing clones were sequenced using M13F primers (Table 2.2).

3. Acclimation of Bacterial Communities to 2-Chloropropionic Acid (2MCPA)

3.1 Introduction

Halogenated compounds are widely produced industrially and their biodegradation by microorganism is of great interest, particularly with regard to bioremediation. 2-Chloropropionic acid (2MCPA) is a short-chain α-chlorinated haloacids (αHA), produced industrially and used as intermediate in manufacturing of herbicides such as chlorophenoxypropionic acids (Jones et al., 1992; Kurihara and Esaki, 2008). It contains a chiral carbon atom producing two enantiomeric forms (D- and L-isomers).

This compound can serve as carbon and energy source to support bacterial growth, and many bacterial species capable of mineralising this compound have been described (Slater et al., 1979; Janssen et al., 1985). Biodegradation of αHAs by microorganisms requires the production of dehalogenases that catalyze the cleavage of carbon-halogen bond (Slater et al., 1979). The αHA dehalogenases have been divided into two evolutionary unrelated groups; groupI (*dehI*) and groupII (*dehII*) dehalogenases (Hill et al., 1999). These groups or families of dehalogenases catalyze hydrolytic dehalogenation of αHAs via different reaction mechanisms (Hill et al., 1999; Kurihara and Esaki, 2008; Schmidberger et al., 2008).

Most recent studies investigating αHA biodegradation and diversity of dehalogenase containing bacterial populations have been restricted to the use of cultivation-dependent methods which depend on isolating bacterial strains (Goldstein et al., 1985; Wagner et al., 1993). These methods are subject to cultivation bias and changes occurring during acclimation of the bacterial populations throughout degradation of the substrates are not usually considered. Relatively few studies have used cultivation-independent molecular based approaches; for example, study performed by Marchesi and Weightman (2003b) comparing the dehalogenase gene pool in bacteria enriched and isolated on 2,2-dichloropropionic acid (22DCPA), which showed that 16S rRNA and catabolic (*deh*) genes obtained by cultivation were different from those obtained by cultivation-independent methods. It is apparent that the isolated strains might not be those that perform the same task in the environment. For example, PNP-utilizing *Pseudomonas* sp., isolated from the enrichment, did not mineralise PNP in sample of lake water (Goldstein et al., 1985).

Studies using rRNA approaches have clearly demonstrated that many bacteria present in environment have not yet been cultivated or isolated, and that bacterial diversity in natural environment is much greater than diversity of bacteria that have been obtained by cultivation (Ward et al., 1990; Muyzer et al., 1993; Wagner et al., 1993; Snaidr et al., 1997; Juretschko et al., 1998). Thus many bacterial species remain uncharacterized because they are unable to grow under the laboratory conditions on conventional culture media (Amann et al., 1995; Leadbetter, 2003). Despite the fact that cultivation-dependent approaches are important for understanding the physiological potential of isolated organism, they do not provide comprehensive information on the composition of microbial communities (Wintzingerode et al., 1997; Marchesi and Weightman, 2003b; Van Hamme et al., 2003). Therefore, molecular techniques, based on analysis of DNA and RNA extracted directly from the environment, have been used for identification of bacteria, since this removes cultivation bias.

The Ready Biodegradation Test (RBT) is a model OECD test of biodegradability. In this study, a model pollutant, the αHA 2-monochloropropionic acid (2MCPA) was enriched, using activated sludge or soil. Generally, activated sludge is used as inoculum in the RBTs to assess biodegradability of chemicals, since chemicals which enter the environment are often discharged through waste water. Soil possesses highly diverse microbial communities that include many species able to degrade organic pollutants. Activated sludge is a complex microbial community in waste water treatment plant, and it is dominated by members of *Proteobacteria* (particularly the Alpha, Beta, and Gamma classes) (Wagner et al., 1993), *Actinobacteria* and *Bacteroidetes* (Wagner and Loy, 2002). The microbial composition of samples of activated sludge differ from one plant to another and is affected by many factors including sewage water quality, methods of collection and storage, seasons, and other environment factors (Tabka et al., 1993; Forney et al., 2001). These differences affect the outcome biodegradability tests and can result in inconsistency (Forney et al., 2001).

3.2 Aims

The aim of the work described in this chapter was to use cultivation-independent methods to investigate the bacterial community adaptation to 2MCPA degradation, and to characterize bacterial community structure and changes occurring during acclimatization and degradation of 2MCPA in an RBT system. Activated sludge (at the standard OECD concentration) and different soil inoculum concentrations (0.10% and 0.05% w/v) were used to inoculate RBTs containing 2MCPA as sole carbon source, to evaluate the effect of inoculum size and type on community enrichment and RBT outcome. Separation of 16S rRNA gene PCR amplicons using DGGE was used to observe changes in the RBTs microbial community during enrichment with 2MCPA, and the enrichment of dehalogenase genes was monitored using degenerate *deh* primers (Hill et al., 1999).

3.3 Results

3.3.1 Biodegradation of 2MCPA by microbial communities in RBTs using activated sludge or soil (0.10 or 0.05%) as an inoculum

Lyophilised activated sludge (0.02%) and soil samples (0.10 or 0.05%) were used in separate experiments to inoculate RBTs containing 2MCPA (20 mgC/l) as the sole source of carbon and energy. 2MCPA was totally dechlorinated in lyophilised activated sludge test after 18 days following a lag phase approximately 10 days (Figure 3.1). The two soil inoculated RBTs showed total dechlorination after 8 and 60 days for 0.10% and 0.05%, with lag phases of 4 and 8 days, respectively (Figure 3.1). 2MCPA dechlorination in the LS-RBT gave a biphasic curve: phase 1 started at day 8 and plateaued around day 14; and phase 2 started at day 37 to total dechlorination by day 60 (Figure 3.1). There was a period of about 20 days (from day 17 to day 37) when the rate of 2MCPA dechlorination (after 50%) was significantly slower.

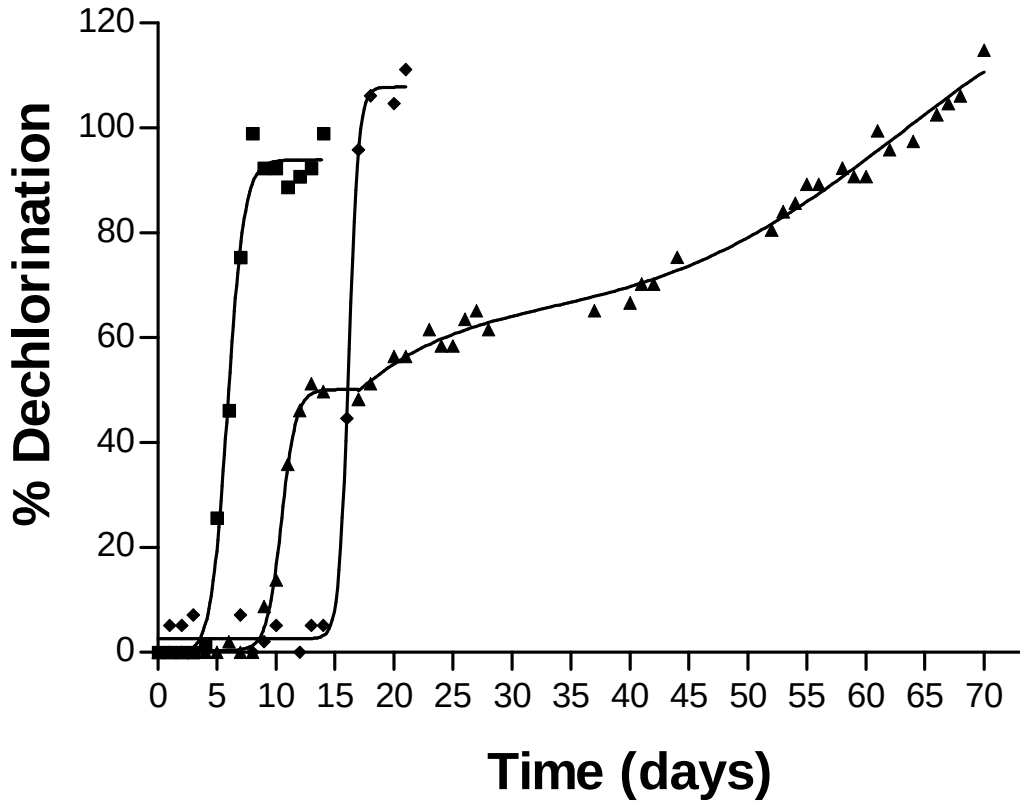

Figure 3.1 Degradation curves for RBT experiments conducted with 0.02% activated sludge ◆ (AS-RBT); 0.10% soil ■ (HS-RBT); 0.05% soil ▲ (LS-RBT). The RBTs contained 0.55 mg/l (20 mg C/l) 2MCPA, and were incubated at 20°C under aerobic conditions.

3.3.2 16S rRNA gene PCR-DGGE analysis of bacterial community changes in 2MCPA RBT inoculated with activated sludge (AS-RBT)

Samples from selected time points were chosen from the RBTs to investigate community change during 2MCPA degradation using 16S rRNA gene PCR-DGGE. In addition, selected time points from the RBTs were assayed for presence of *dehI* and *dehII* genes by PCR using the degenerate primers described by Hill et al. (1999). The time points for DNA extraction were chosen to reflect the stages of 2MCPA degradation reflected in the degradation curves (Figure 3.1).

Denaturing gradient gel electrophoresis (DGGE) of the 16S rRNA genes was used to assess the diversity of the bacterial community during 2MCPA degradation in the RBTs. Changes in DGGE banding patterns (or profile) reflected changes in the composition of the bacterial community, and diversity of bacterial community was reflected by the number of DGGE bands.

In general, during enrichment, the bacterial community became less diverse, and some bands became more dominant. Phylotypes represented by bands AS1, AS2, AS3, AS4 (Figure 3.2; Table 3.1) were present in the starting population (day 0) and they disappeared during the degradation of 2MCPA. DGGE profiles from AS-RBT (Figure 3.2) showed that different phylotypes were enriched during 2MCPA degradation. Band AS6 appeared at day 11, in conjunction with the beginning of 2MCPA dechlorination (Figures 3.1 and 3.2). Bands AS7, AS8, AS9, AS10 appeared between days 11 and 16 (Figures 3.1 and 3.2) and persisted to day 20. None of these bands were detected in the starting population (day 0). Analysis of the partial 16S rRNA sequence from excised bands identified AS6 as close relative of the *Caulobacter* sp. (*Alphaproteobacteria*). Band AS7 and AS9 were identical and most closely related to *Cupriavidus* sp. (*Betaproteobacteria*), and band AS10 was closely related to *Myxobacterium* (*Deltaproteobacteria*) (Table 3.1). Band AS8 was also present at days 16 and 20, but no sequence could be obtained from this band. Re-amplified PCR product of band AS8 run on DGGE gel with the day 20 samples of AS-RBT (Figure 3.2) (see section 2.8), and the result showed that band AS8 which appeared as single dense band, contained multiple bands (populations) that co-migrated to the same position in the gel (Figure 3.3) (Joynt et al., 2006). Therefore, it was excluded from further analysis. Previously it has been reported that some highly similar 16S rRNA

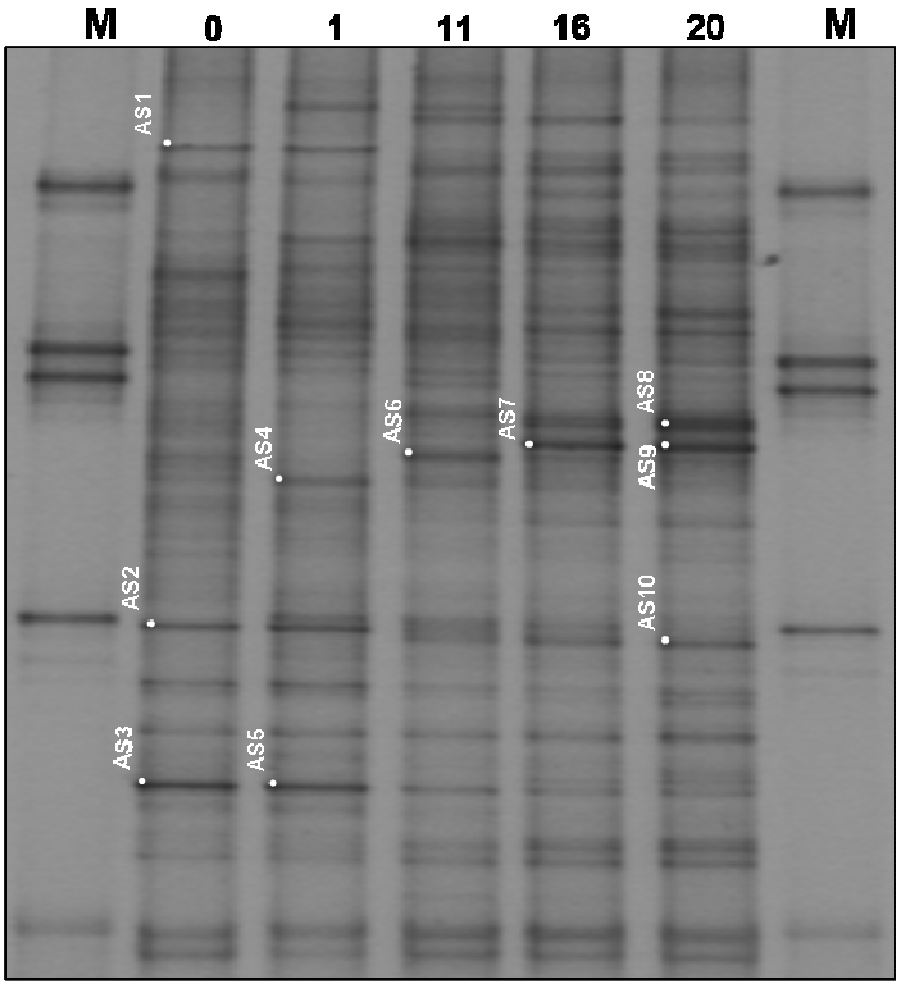

Figure 3.2 DGGE profile of bacterial 16S rRNA gene sequences obtained by PCR with DNA extracted from activated sludge RBT (AS-RBT) in the presence of 2MCPA using 357FGC-518R primers. AS numbers designations indicate bands excised for sequencing. Numbers above each lane represent the day of sampling (see Figure 3.1). M = marker.

Table 3.1 Closest 16S rRNA matches for excised DGGE bands from the activated sludge RBT using BLASTN and the ribosome database project (RDP).

DGGE band ID[a,b]	Nearest match using NCBI nucleotide BLAST	Sequence similarity (%)	Alignment length (bp)	Phylogenetic affiliation	Nearest cultured relative using RDP sequence match	S-ab score[c] (%sequence similarity)
AS1	Poor sequence					
AS2	Uncultured bacterium (EU539613)	100	174	*Actinobacteria*	*Janibacter limosus* (Y08539)	1.000 (100)
AS3, 5	*Micropruina glycogenica* (AB012607)	99	169	*Actinobacteria*	*Micropruina glycogenica* (AB012607)	0.951 (99)
AS4	*Thiothrix eikelboomii* (L79965)	97	170	*Gammaproteobacteria*	*Thiothrix eikelboomii* (AB042819)	0.941 (97)
AS6	*Caulobacter* sp. (EU857422)	100	169	*Alphaproteobacteria*	*Caulobacter fusiformis* (AJ007803)	1.000 (100)
AS7, 9	*Cupriavidus* sp. (AB266610)	97	194	*Betaproteobacteria*	*Cupriavidus* sp. (AB266610)	0.882 (97)
AS8	Poor sequence					
AS10	Uncultured bacterium (AB286342)	89	194	*Deltaproteobacteria*	*Myxobacterium* (AB246772)	0.628 (87)

a. See Figure 3.2
b. Bands grouped together shared 100% sequence identity
c. As defined by Woese (1987)

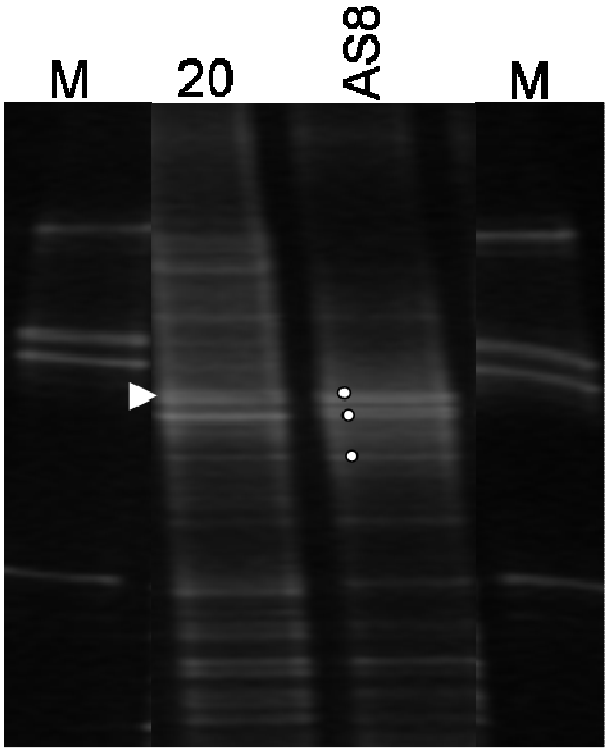

Figure 3.3 DGGE profile of 16S rRNA gene by PCR using 357FGC-518R primers. Lane labelled 20, represent sample of day 20 of AS-RBT refer to Figure 3.2. Lane labelled AS8, represent re-amplification of the band AS8 which originated from day 20 sample of the AS-RBT refer to figure 3.2. Circles show the multiple bands amplified from band AS8 shown by a white arrow. M = marker.

gene sequences might remain undistinguished throughout the DGGE analysis because of sequencing problems (Gonzalez and Sainz-Jimenez, 2004).

3.3.3 16S rRNA gene PCR-DGGE analysis of bacterial community changes in 2MCPA RBT inoculated with 0.10% soil (HS-RBT)

DGGE profiles of the HS-RBT (Figure 3.4A) showed five phylotypes were clearly enriched during 2MCPA degradation. The closest sequence matches for all bands labelled in Figure 3.4A are shown in Table 3.2. Bands HS2, HS3, HS6, HS7, and HS8 (Figure 3.4A; Table 3.2) were enriched by day 7 and persisted through to day 12, none of these were detected in the starting population (day 0). Analysis of the 16S rRNA gene sequences of these bands showed that they were closely related to *Herbaspirillum* sp. (*Betaproteobacteria*). A phylotype represented by band HS1 (Figure 3.4A, Table 3.2), also not present in the starting population, was enriched by day 7 and persisted through to day 8 during 2MCPA enrichment. Analysis of the partial 16S rRNA gene sequences of the excised band showed that the HS1 was related to *Herminiimonas fonticola* (*Betaproteobacteria*). A strain of *Herminiimonas* sp. was isolated from a water treatment plant able to degrade MCA containing dehalogenases (Zhang et al., 2009b). 16S rRNA analysis of the partial 16S rRNA gene sequences of the two phylotypes represented by HS4 and HS5 showed that they were closely related to *Acidovorax* sp. (*Betaproteobacteria*). Band HS9 appeared at days 8 and 12, but was not present in the day 7 community and represented a phylotype closely related to *Brevundimonas* sp. (*Alphaproteobacteria*).

3.3.4 16S rRNA gene PCR-DGGE analysis of bacterial community changes in 2MCPA RBT inoculated with 0.05% soil (LS-RBT)

DGGE profiles (Figure 3.4B) showed that different phylotypes were enriched in the LS-RBT during dechlorination of 2MCPA. The closest relative of the dominant DGGE bands shown in Figure 3.4B are summarized in Table 3.3. DGGE profile (Figure 3.4B) showed that three different phylotypes were enriched during dechlorination of 2MCPA. Also, to some extent, the profiles reflected the biphasic degradation curve observed in this enrichment (Figure 3.1). Bands LS1 and LS4 appeared during phase 1 at day 10 and gradually faded after day 17 (Figures 3.1 and 3.4B). Bands LS7 and LS8 appeared during phase 2 at day 37 and persisted through to

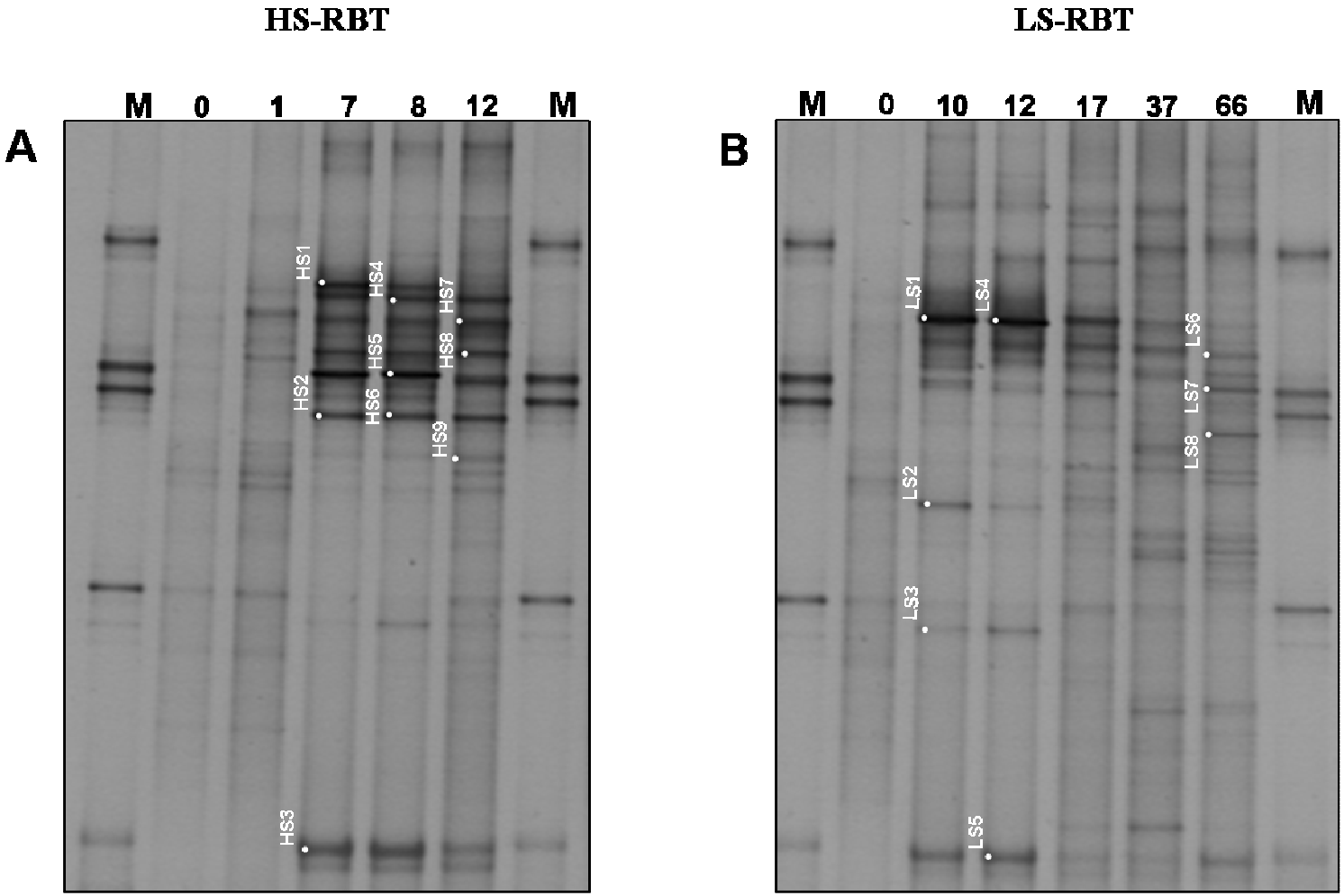

Figure 3.4 DGGE profile of bacterial 16S rRNA gene sequences obtained by PCR with DNA extracted from soil RBTs in the presence of 2MCPA using 357FGC-518R primers. (A) 16S rRNA profile from 0.10% soil RBT (HS-RBT). (B) 16S rRNA profile from 0.05% soil RBT (LS-RBT). HS and LS numbers designations indicate bands excised for sequencing. Numbers above each lane represent the day of sampling (see Figure 3.1). M = marker.

Table 3.2 Closest 16S rRNA matches for excised DGGE bands from the 0.10% soil RBT (HS-RBT) using BLASTN and the ribosome database project (RDP).

DGGE band ID[a,b]	Closest match using NCBI nucleotide BLAST	Sequence similarity (%)	Alignment length (bp)	Phylogenetic affiliation	Nearest cultured relative using RDP sequence match	S-ab score[c] (%sequence similarity)
HS1	*Herminiimonas* sp. (GU932947)	98	194	*Betaproteobacteria*	*Herminiimonas fonticola* (AY676462)	1.000 (98)
HS2, 6	Uncultured Oxalobacteraceae bacterium (AY624616)	97	194	*Betaproteobacteria*	*Herbaspirillum frisingense* (AJ238358)	0.956 (96)
HS3	Uncultured Proteobacterium (EF196962)	94	194	*Betaproteobacteria*	*Herbaspirillum lusitanum* (AF543312)	0.978 (94)
HS4	Uncultured bacterium (AM909976)	94	194	*Betaproteobacteria*	*Acidovorax* sp. (AB076843)	0.848 (91)
HS5	Uncultured Comamonadaceae bacterium (EU642288)	100	194	*Betaproteobacteria*	*Acidovorax* sp. (Y18617)	1.000 (100)
HS7	*Herbaspirillum* sp. (HQ728570)	91	193	*Betaproteobacteria*	*Herbaspirillum seropedicae* (AJ238361)	1.000 (92)
HS8	*Herbaspirillum* sp. (AJ430686)	100	194	*Betaproteobacteria*	*Herbaspirillum frisingense* (AJ238358)	0.973 (99)
HS9	Alphaproteobacterium (EF612346)	93	194	*Alphaproteobacteria*	*Brevundimonas* sp. (AJ227799)	0.846 (93)

a. See Figure 3.4A
b. Bands grouped together shared 100% sequence identity
c. As defined by Woese (1987)

Table 3.3 Closest 16S rRNA matches for excised DGGE bands from the 0.05% soil RBT (LS-RBT) using BLASTN and the ribosome database project (RDP).

DGGE band ID[a,b]	Closest match using NCBI nucleotide BLAST	Sequence similarity (%)	Alignment length (bp)	Phylogenetic affiliation	Nearest cultured relative using RDP sequence match	S-ab score[c] (%sequence similarity)
LS1, 4	Uncultured bacterium (AY955094)	98	194	*Betaproteobacteria*	*Comamonas testosteroni* (AB064318)	0.862 (94)
LS2	Uncultured Polaromonas sp (EF674513)	100	194	*Betaproteobacteria*	*Polaromonas aquatica* (AM039830)	1.000 (100)
LS3	Uncultured bacterium (AY955094)	96	194	*Betaproteobacteria*	*Variovorax* sp. (AM411933)	0.879 (95)
LS5	Uncultured bacterium (AY955094)	98	194	*Betaproteobacteria*	*Comamonas testosteroni* (AY247415)	0.862 (94)
LS6	Uncultured bacterium (AB280395)	94	194	*Betaproteobacteria*	*Herbaspirillum* sp. (AB366209)	0.852 (91)
LS7	Poorsequence					
LS8	*Methylibium petroleiphilum* PM1(CP000555)	100	194	*Betaproteobacteria*	*Methylibium petroleiphilum* PM1 (AF176594)	1.000 (100)

a See Figure 3.4B
b. Bands grouped together shared 100% sequence identity
c. As defined by Woese (1987)

day 66 (Figures 3.1 and 3.4B). None of these bands were detected in the starting population (day 0). Analysis of the partial 16S rRNA sequences of excised band identified LS1 and LS4 identical, and closely related to *Comamonas* sp. (*Betaproteobacteria*; Table 3.3). move to discussion Song et al. (2004) reported isolation of a strain of *Comamonas* sp. from waste water able to degrade 2MCPA. 16S rRNA gene sequences analysis of LS8 showed that this band represented a phylotype closely related to *Methylibum petroleiphilum* (Nakatsu et al., 2006). Band LS6 (Figure 3.4B) was closely related to *Herbaspirillum* sp. (*Betaproteobacteria*). 16S rRNA sequence analysis of LS3 showed it represented an organism closely related to *Varivorax* sp. (*Betaproteobacteria*; Table 3.3). Figure 3.5 includes two additional time points (days 58 and 61) to show the appearance of *Comamonas* sp. (LS1) with onset of degradation of 2MCPA, as well as the enrichment of *Methylibium* sp. (LS8) after day 37 and relation to the biphasic dechlorination curve which papered during degradation of 2MCPA in the LS-RBT (Figure 3.5).

The only 16S rRNA sequence found in both soil RBTs (HS8 and LS6; Figures 3.4A, B), gave 98% similarity to each other and both were assigned to the genus *Herbaspirillum*, which has previously been reported to contains αHA-degrading bacteria (Marchesi and Weightman, 2003a).

3.3.5 Presence of αHA dehalogenase genes (*dehI*, *dehII*) in the activated sludge RBT (AS-RBT)

Samples from days 0, 1, 11, 16, and 20 from the AS-RBT were tested for presence of group I and group II α-HA dehalogenase genes (*dehI*, *dehII*) using primers described by Hill et al. (1999). Samples from day 11, 16 and 20 gave positive PCR products with groups I *deh* gene primers ($dehI_{for1}$ / $dehI_{rev1}$; 230 bp), whereas DNA from the starting population (day 0) was negative for *dehI* (Figure 3.6A). PCRs with the alternative *dehI* primers, which amplify a larger product (504 bp), produced artifact bands but failed to detect *dehI* genes in any of the samples, even though the positive control was clearly amplified (Figure 3.6B). All DNA samples extracted on days 0, 1, 11, 16 and 20 gave PCR products with group II *deh* genes primers (422 bp); however PCR product at days 16 and 20 were at a significantly higher level (Figure 3.6C).

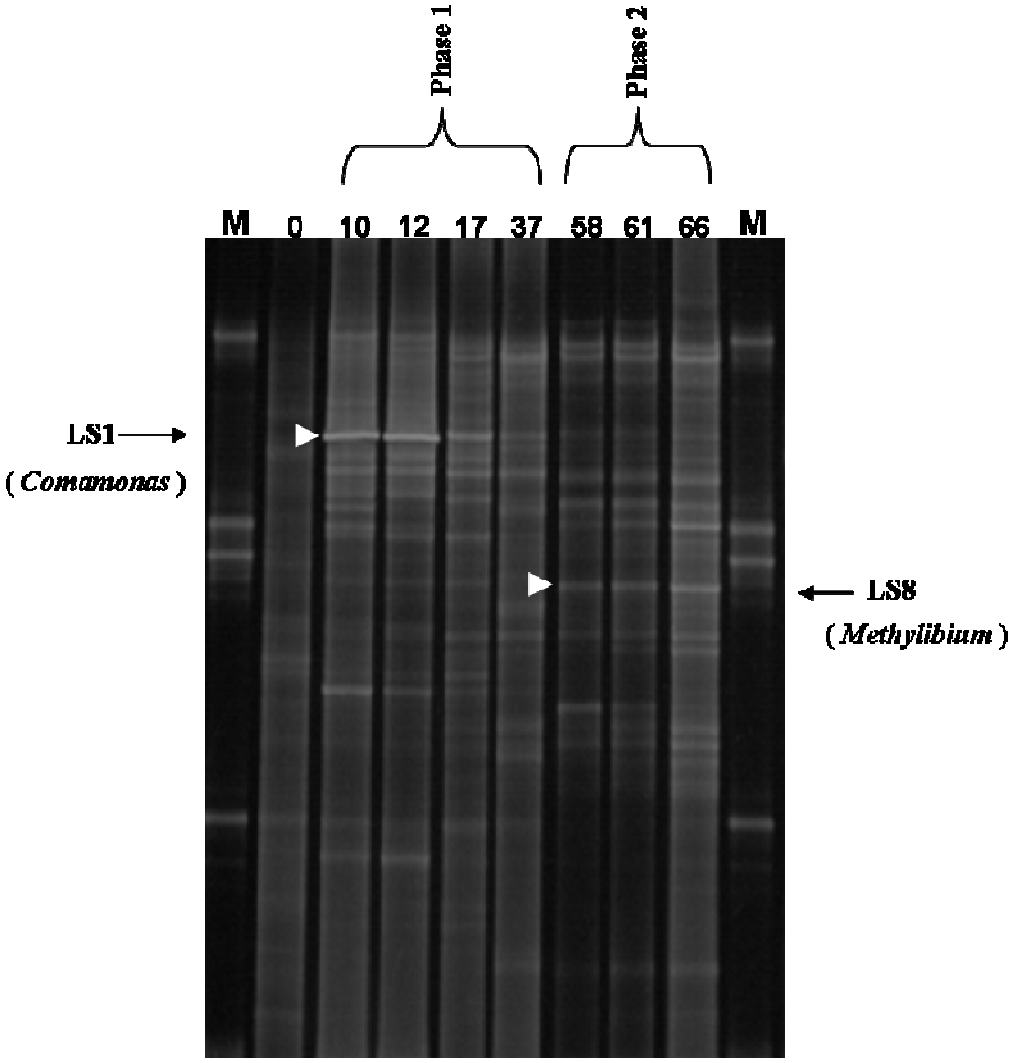

Figure 3.5 DGGE profile of bacterial 16S rRNA gene sequences of the same samples of Figure 3.4B; LS-RBT, with two more samples; days 58 and 61. Shows the appearance of *Comamonas*-like phylotype (LS1) with onset of 2MCPA degradation and enrichment of *Methylibium*-like phylotype (LS8) after day 37, indicated by white arrows, in relate to biphasic dechlorination curve during degradation of 2MCPA. Numbers above each lane represent the day of sampling (see Figure 3.1). M = marker.

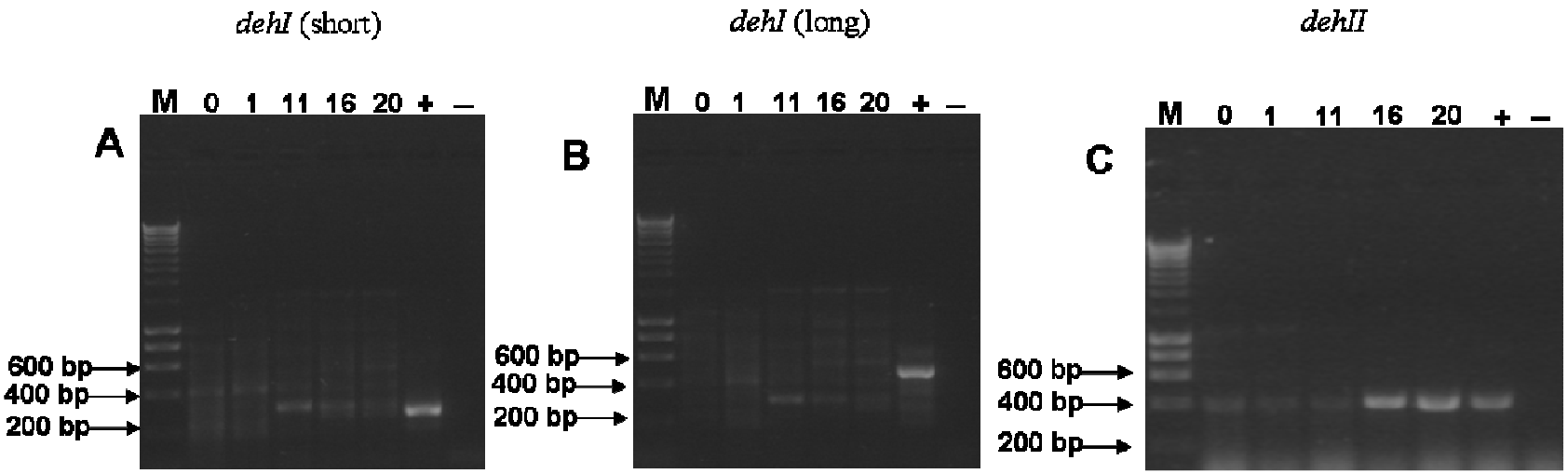

Figure 3.6 Amplification of dehalogenase genes by PCR from activated sludge (AS) RBT. (A) *dehI* (short; $dehI_{for1}/dehI_{rev1}$) gene PCR. (B) *dehI* (long; $dehI_{for1}/dehI_{rev2}$) gene PCR (C) *dehII* gene PCR (see Table 2.2). Numbers above each lane represent the day of sampling (see Figure 3.1); M = marker; + = positive PCR control; - = negative PCR control. Numbers on side indicate DNA fragment size.

3.3.6 Presence of αHA dehalogenase genes (*dehI*, *dehII*) in the soil RBTs (HS-RBT and LS-RBT)

Samples from both soil RBTs (0.10%, HS and 0.05%, LS) were screened for presence of *dehI* and *dehII* genes using primers described by Hill et al. (1999). DNA samples from day 7, 8, and 12 in the HS-RBT gave positive PCR product with group I *deh* gene ($dehI_{for1}$ / $dehI_{rev1}$; 230 bp) (Figure 3.7A), and group II *deh* gene primers (Figure 3.7C). However, PCR products of *dehI* were at considerably higher level (Figure 3.4A, C). DNA from the starting community (day 0 and day 1) was negative for both *dehI* and *dehII* genes (Figure 3.7A, C). PCRs with the alternative *dehI* primer sets ($dehI_{for1}$/$dehI_{rev2}$; 504 bp) again produced artifact bands and showed no detectable *dehI* genes in any of the samples (Figure 3.7B).

All DNA samples from LS-RBT gave *dehII* PCR product, except starting community (day 0), with strong PCR signal at days 10 and 12 (Figure 3.8C). PCR products of *dehI* gene were obtained at days 37 and 66 with group I *deh* gene primers ($dehI_{for1}$ / $dehI_{rev1}$; 230 bp) (Figure 3.8A). The appearance of *dehII* gene products at day 10, 12 correspond with phase 1 of 2MCPA degradation (Figure 3.1), whereas *dehI* gene was detected at day 37, corresponding to the start of phase 2 of 2MCPA degradation (see Figure 3.1). No PCR products were obtained using the longer *dehI* PCR primer (see Figure 3.8B). Again, DNA from the starting community (day 0) was negative for *dehI* and *dehII* (Figure 3.8A, C).

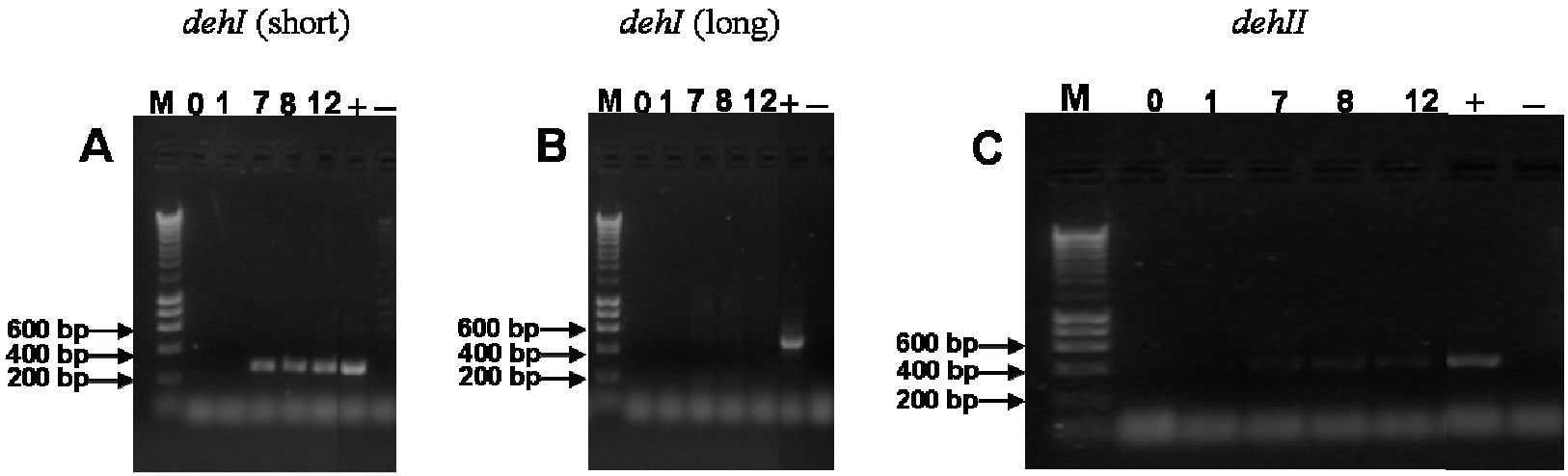

Figure 3.7 Amplification of dehalogenase genes by PCR from 0.10% soil (HS) RBT. (A) *dehI* (short) gene PCR. (B) *dehI* (long) gene PCR. (C) *dehII* gene PCR. Numbers above each lane represent the day of sampling (see Figure 3.1); M = marker; + = positive PCR control; - = negative PCR control. Numbers on side indicate DNA fragment size.

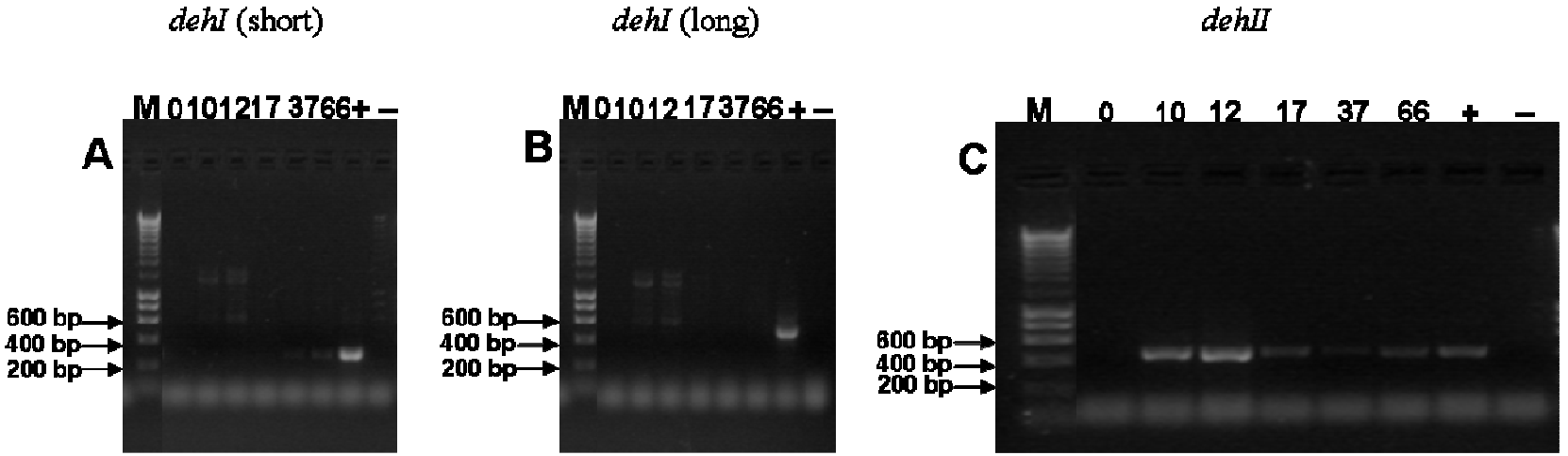

Figure 3.8 Amplification of dehalogenase genes by PCR from 0.05% soil (LS) RBT. (A) *dehI* (short) gene PCR. (B) *dehI* (long) gene PCR. (C) *dehII* gene PCR. Numbers above each lane represent the day of sampling (see Figure 3.1); M = marker; + = positive PCR control; - = negative PCR control. Numbers on side indicate DNA fragment size.

3.4 Discussion

3.4.1 Bacterial community changes during 2MCPA degradation in activated sludge and soil RBTs

2MCPA was readily and completely dechlorinated in all of the RBTs, whether inoculated with lyophilized activated sludge (AS-RBT) or soil (HS- and LS-RBT) (Figure 3.1). In the RBT inoculated with 0.05% soil (LS-RBT) degradation was slower and occurred in two distinct phases (Figure 3.1).

Using the cultivation-independent approach, it was possible to follow the enrichment of different phylotypes and corresponding *deh* genes in the RBTs. All three RBTs underwent significant changes in community structure, following exposure to 2MCPA (Figures 3.2 & 3.4), associated with changes in αHA dehalogenase genes *dehI* and *dehII* (Figures 3.6, 3.7 & 3.8). 16S rRNA PCR-DGGE based bacterial community analysis of the RBTs revealed enrichment of different phylotypes, representing different species that were either not present or not dominant in the inoculant community (at t = 0).

Generally, three different patterns in DGGE profiles were observed. Firstly, some phylotypes were either unaltered or disappeared during enrichment. Secondly, phylotypes appeared during enrichment that were not evident or dominant in the starting community, and persistent until the experiment finished. Thirdly, some phylotypes appeared transiently during enrichment.

Phylotypes that were unaltered or disappeared probably represented organisms that were not involve in 2MCPA degradation. The dominant 16S rRNA gene fragments presented in the staring population of the AS-RBT (e.g. AS1,AS2, AS3 and AS5, Figure 3.2; Table 3.1) represent bacteria commonly found in activated sludge (Hill and Weightman, 2003). For example, *Micropruina glycogenica* (AS3, Figure 3.2; Table 3.1) a Gram-positive species of *Actinobacteria*, has previously been isolated from activated sludge showing phosphorus removal activity (Shintani T, 2000).

Phylotypes that appeared during enrichment and substrate degradation (e.g. AS7, AS8, AS10, Figure 3.2; Table 3.1) coinciding with appearance of *dehI* and *dehII* genes (Figure 3.4A,C), such as the *Cupriavidus* and *Myxobacterium*-related species

seen in the AS-RBT experiment, were implicated in the degradation of the 2MCPA. *Cupriavidus* and *Myxobacterium* strains that are capable of degrading 2,4-dichlorophenoxyacetic acid have previously been isolated from soil (Ka et al., 1994; Sakai et al., 2007). *Cupriavidus* have been reported to be able to degrade chlorinated compounds, such as chlorophenol, 2,4-D, 3-chlorobenzoate, and 4-methyl-2-chlorophenoxy acetate (Pérez Pantoja et al., 2008). The genus *Cupriavidus* and the closely related *Betaproteobacterium Ralstonia* are well known to be catabolically versatile (Vandamme and Coenye, 2004; Vaneechoutte et al., 2004; Lykidis et al., 2010), and αHA-degrading members of the genus *Ralstonia* have been isolated and reported to contain *dehI* and *dehII* genes (Hill and Weightman, 2003).

In the HS-RBT, the dominant phylotypes were assigned to *Herbaspirillum* species which were enriched during 2MCPA degradation, and coincided with presence of *dehI* and *dehII* genes (HS2, HS3, HS7, HS8, Figure 3.4A & 3.7A, C). *Herbaspirillum* sp. strains have previously been reported to degrade chlorinated pollutants, including several different αHAs (Hill and Weightman, 2003; Marchesi and Weightman, 2003a). *Acidovorax* sp. (HS4, HS5, Figure 3.4A; Table 3.2), a genus reported to degrade halobenzoate compounds (Song et al., 2000), also appeared in the HS-RBT.

A *Comamonas* sp. (LS1, Figure 3.4B; Table 3.3), was enriched in the LS-RBT coinciding with the presence of *dehII* gene (Figure 3.8C). Schwarze et al. (1997) reported isolation of *Comamonas acidovorans* strain RS7, which utilized 22DCPA, and showed this isolate was able to grow on MCA, DCA, and 2MCPA owing to production of L-isomer specific dehalogenases. A *Methylibium petroleiphilum* (LS8, Figure 3.4B; Table 3.3), was enriched in the LS-RBT coinciding with the presence of *dehI* gene (Figure 3.8A). Previously, *Methylibium petroleiphilum* has not been reported to contain αHA dehalogenase genes, but strains have been reported to degrade environmental pollutants such as methyl tert-butyl ether (Nakatsu et al., 2006).

Some DGGE bands appeared transiently in the RBTs (e.g. AS4, AS6, HS1, LS1, LS2, LS5; Figures 3.2 & 3.4). Appearance of dominant, but transient phylotype AS6, representing *Caulobacter*-related species, coincided with the amplification of *dehI* and *dehII* genes in the activated sludge RBT at day 11 (Figure 3.2 & 3.6A,C), a possible explanation is that this organism was producing both types of dehalogenase

and was responsible for 2MCPA dechlorintion. *Caulobacter* sp. strains capable of degrading αHAs (22DCPA), and containing *dehI* gene and *dehII* have been previously reported (Marchesi and Weightman, 2003b). *Caulobacter* spp. have also been isolated from oil spill contaminated sea in Japan (Nakamura et al., 2007) and shown to be able to degrade phenol (Fischer et al., 2010) and 2-nitrobenzoate (Iwaki and Hasegawa, 2007). A *Comamonas* related phylotype also appeared transiently in the LS-RBT (LS1, LS4, LS5, Figure 3.4B; Table 3.3), coinciding with the presence of strong PCR signal of *dehII* gene (Figure 3.8C). Members of the genus *Comamonas* have been reported previously being able to degrade αHA (22DCPA), expressing a single dehalogenase (Schwarze et al., 1997).

Disappearance of some bacteria towards the end of 2MCPA degradation could be a result of the failure of the bacterium to survive (i.e. they are able to grow at high 2MCPA concentration and when substrate concentration is low they may out-competed by others), or they may only degrade 2MCPA to some extent as they only have one dehalogenase which is able to act on one isomer of the substrate.

Phylotype identities indicated that a greater diversity of bacterial species was enriched than have been reported in previous studies investigating 2MCPA degradation based on cultivation and isolation. The αHA dehalogenating organism studied to date are mostly *Proteobacteria* (McCaig et al., 2001; Marchesi and Weightman, 2003b, a), and in this study all of the 16S rRNA phylotypes enriched by 2MCPA were assigned to the phylum *Proteobacteria* (Tables 3.1, 3.2, and 3.3). The explanation may be that the enrichment methods (batch culture) are selective for groups belonging to the *Proteobacteria* and they grow most easily under conditions which were selecting against less competitive potential αHA-degrading bacteria from other phyla. Alternatively, it might reflect biases in the DNA extraction (Wintzingerode et al., 1997; Nakatsu, 2007). However, previous studies indicated dominance of members of *Proteobacteria* in activated sludge inocula (Wagner et al., 1993; Snaidr et al., 1997), and so it is not inconsistent that results from the present study show that *Proteobacteria*, widely distributed in activated sludge and soil, were enriched by 2MCPA.

Although, PCR-DGGE is not a quantitative method, band intensities reflect phylotype abundance to some extent. The correlation of dominant DGGE bands with *deh* genes

may infer enrichment of specific dehalogenase containing species. However, it is possible for relatively low abundance species or phylotypes to be highly active and responsible for 2MCPA degradation (Nakatsu, 2007).

Comparisons betwen the HS-RBT and LS-RBT showed that decreasing the inoculum concentration in the soil RBTs affected the dechlorination of 2MCPA by increasing the time required to achieve total dechlorination of the substrate; 60 days in LS-RBT compared to 12 days in HS-RBT (Figure 3.1). The LS-RBT underwent a secondary lag phase lasting 20 days. Other studies have also shown increasing lag phase with decreasing inoculum size (Spain et al., 1980; Ramadan et al., 1990; Ingerslev et al., 2000). A decrease in inoculum concentration would reduce the number of specific αHA-degrading bacteria initialy present in the RBT, which would increase the time for cell division to produce a strong degradation population (Spain et al., 1980; Mattes et al., 2010). The bacterial phylotypes and dehalogenases enriched in the LS-RBT were different from those in the HS-RBT (Figure 3.4A; Table 3.2, Figure 3.4B; Table 3.3). Franklin et al. (2001) reported that dilution of environmental samples significantly altered the community structure and diversity. The decrease in inoculum concentration may have eliminated the faster growing organisms that were present at the higher inoculum density, and resulted in enrichment of other slower growing organisms, such as *Methylibium* related phylotypes.

The biphasic degradation curve in the LS-RBT experiment was associated with changing of population following 50% dechlorination (Figures 3.1 & 3.4B). This suggests that the population change was related to the observed biphasic curve and the dechlorination of 2MCPA. Phase 1 (days 10-17; Figure 3.1) was associated with the enrichment of *Comamonas*-related organism (LS1, Figure 3.4; Table 3.3), whereas, phase 2 (days 37-66; Figure 3.1) was associated with the enrichment of the *Methylibium petroleiphilum* phylotype (Figure 3.4B; Table 3.3).

The changes observed in the bacterial community structure, following 50% dechlorination in the LS-RBT also coincided with a change in dehalogenase genes (Figure 3.8). Enrichment of *dehII* prior to *dehI* in the LS-RBT most likely reflects the environmental abundance of the two families, since *dehII* genes have been shown to be more diverse and widely distributed than *dehI* genes (Marchesi and Weightman, 2003b). The low soil inoculum used in the LS-RBT may have caused a reduction in

an already small *dehI*-containing bacterial community, and, therefore, the second lag phase may represent the time taken to enrich a *dehI*-containing organism from a very small starting population.

Changes in the bacterial community and dehalogenase genes, around 50% dechlorination in the LS-RBT, suggest a link to the racemic nature of 2MCPA and the enantioselectivity of αHA dehalogenases (DehI and DehII). As previously reported, members of the DehI family can act on both D- and L-2MCPA isomers, or D-2MCPA only (Weightman et al., 1982; Leigh et al., 1988; Schmidberger et al., 2008), whilst DehII dehalogenases are only able to utilize the L-isomer (Liu et al., 1994; Hill et al., 1999). This suggests that in the LS-RBT L-2MCPA was initially degraded by DehII, leaving the D-2MCPA only in the test system, and then organisms containing DehI completed the degradation of the remaining D-2MCPA.

Previous studies have reported the greater abundance and diversity of group II *deh* genes over group I *deh* genes in the environment (Hill et al., 1999). In this study *dehII* gene amplification was observed in the starting population (day 0) of some RBTs prior to any enrichment (Figures 3.6C), but there was no detectable *dehI* (Figures 3.6A, 3.7A and 3.8A). DehI dehalogenases are not related to any other protein families (Schmidberger et al., 2008); however, the DehII family is recognized to belong to a large superfamily of hydrolase-encoding gene, designated the HAD superfamily (Koonin and Tatusov, 1994). Amplification of group II *deh* genes for the AS-RBT prior to enrichment of 2MCPA-degrading bacteria may indicate that they contained *dehII* dehalogenase genes, but may also have been the result of the presence of group II *deh*-like genes of HAD superfamily; i.e. other hydrolases, but not necessarily dehalogenases.

None of the RBTs gave positive PCR product using the longer *dehI* gene primer set (Figures 3.6B, 3.7B, 3.8B), even though clear PCR products were obtained using the short *deh* primer pairs (Figure 3.6A, 3.7A, 3.8A). Other studies have reported similar results; for example, the *dehI* gene in a strain of *Xanthobacter autotrophicus* (Marchesi and Weightman, 2003b).

4. Isolation and Characterization of Dehalogenase-Containing Bacteria from Soil and Activated Sludge

4.1 Introduction

Increasingly, cultivation-independent methods are widely used in preference to conventional cultivation techniques for microbial community analysis. It is generally accepted that <1% of bacteria present in the natural environment can be detected by culturing methods (Amann et al., 1995; Neef et al., 1998; DeLong and Pace, 2001; Brinkmeyer et al., 2003; Rappé and Giovannoni, 2003; DeLong et al., 2006) , and the use of molecular techniques has resulted in the discovery of many new bacterial species and higher taxa, as well as the identification of new functional genes (Brinkmeyer et al., 2003; Rappé and Giovannoni, 2003; Junca and Pieper, 2004; Pester et al., 2004; Sato et al., 2005; Jones and Marchesi, 2007; Liu et al., 2011). Although, molecular genetic approaches remove cultivation bias and can be used to some extent for analyzing functional and physiological traits of microbes in the environment, DNA based methods can not be used to measure cellular activity (Lebaron et al., 2001), and may be misleading since many bacteria exist in dormant forms (Kell et al., 1998). It has been shown that there is a positive correlation between activity and cultivability (Soderberg and Baath, 1998), and it is important to measure activity so that environmentally relevant bacteria are assessed and not inactive cells that do not contribute to ecosystem function (Ellis et al., 2003). However, the large majority of microorganisms remain uncharacterized, and even though cultivation techniques are improving, scientific knowledge of their growth conditions in nature, the chemistry of the original environment, life in complex communities, obligate interactions with other organisms, etc, remains insufficient to cultivate most bacteria (Leadbetter, 2003; Gonzalez and Sainz-Jimenez, 2004).

Dunbar et al. (1997) reported that evaluation of the diversity and distribution of catabolic pathways in nature can be distorted by using enrichment culture techniques, and that the identification and isolation of representative organisms is not straightforward because of these biases. The composition and the artificial conditions inherent in most laboratory media and growth conditions may be responsible for this phenomenon (Zengler et al., 2002). Some workers have proposed and used alternative methods for isolating different microbial species that could not be cultivated by

conventional microbiological techniques (Zengler et al., 2002; Kerr and Marchesi, 2006) and others. Success in cultivation experiments, isolating previously uncultivable soil bacteria, has been reported (Janssen et al., 2002; Zengler et al., 2002). Ellis et al. (2003) reported that plate counting may be a more appropriate method to determine the effects of heavy metals on soil bacteria than cultivation-independent methods. Therefore, a combination of cultivation-dependent and independent approaches was used in this study to investigate bacterial diversity of Ready Biodegradation Test (RBT) based enrichments.

The hydrolytic cleavage of the carbon-halogen bond by bacterial dehalogenases is a key step in degradation of halogenated compounds (Fetzner and Lingens, 1994; Janssen et al., 1994b). Enrichment batch culture is the most common method used for isolation of bacteria able to grow on chlorinated compounds and utilize them as the sole sources of carbon and energy. A large number of bacterial dehalogenases have been described with different substrate specificities and reaction mechanisms (Fetzner 1994, Janssen 1994). The α-halocarboxylic acid (αHA) dehalogenases have been identified and purified from several species of bacteria and been widely studied (Table 1.1). Bacteria able to degrade αHA have been enriched, isolated and identified from both pristine and contaminated environments (Senior et al., 1976; Janssen et al., 1985; Tsang et al., 1988; Smith et al., 1990; Liu et al., 1994; Brokamp et al., 1997; Schwarze et al., 1997; Marchesi and Weightman, 2003a) and their dehalogenases have been characterized in terms of structure and reaction mechanism by crystallography (Ridder et al., 1997; Ridder et al., 1999; Schmidberger et al., 2008). Pure cultures are needed to study physiology, biochemistry, substrate ranges and dehalogenase gene regulation, and are also useful for determining the conditions that should be used in practical treatment systems; e.g. bioremediation (biostimulation, bioaugmentation, etc.).

Previous studies suggest that *Proteobacteria* constitute the majority of known αHA-degrading bacteria because they outcompete other bacteria in standard cultivation media. However, Marchesi and Weightman (2003b) reported that cultivated dehalogenase-producing organisms may not represent environmentally relevant αHAs degrading bacteria. Pollutant degradation studies after performing batch-culture enrichment, have resulted in isolating very limited numbers of bacteria which grow

most rapidly on cultivation media (Dunbar et al., 1996). Also, from the limited cultivation-independent studies it is apparent that almost all αHA degrading bacteria are exclusively *Proteobacteria* (Marchesi and Weightman, 2003a). Gram positive bacteria have been reported as being able to utilize and degrade αHAs (Kerr and Marchesi, 2006; Chiba et al., 2009), but data from further characterization and biochemical analysis of their dehalogenases are limited.

4.2 Aims

The main objective of the work described in this chapter was to enrich and isolate bacteria capable of 2MCPA (2-chloropropionate) biodegradation from the RBTs described in Chapter 3. Enrichments in liquid batch-culture were done at two different substrate concentrations and 2MCPA-degrading bacteria isolated from the enrichment cultures were identified using PCR-DGGE of 16S rRNA gene analysis. An additional objective was preliminary characterization of selected bacterial isolates and their dehalogenases by molecular genetic analysis, enzyme assays and gel zymography. The experimental approach for this part of the work is summarized in Figure 2.1, in relation to the work described in Chapter 3.

4.3 Results

4.3.1 Enrichment of 2MCPA-degrading bacteria from the activated sludge RBT (AS-RBT)

Sub-cultures were prepared from plate washes of the activated sludge RBT (AS-RBT) as detailed in section 2.4.1. Choice of time points (days 11, 16 and 20) for inoculation of sub-cultures (S11, S16, and S20, respectively) was based on extent of the 2MCPA degradation (as determined by Cl^- concentration), and changes in the diversity of population in the RBT (Figures 3.1 and 3.2). Liquid sub-cultures were also monitored by 16S rRNA gene PCR-DGGE profiling and band sequencing.

4.3.1.1 DGGE profiles of the sub-cultures from day 11 (S11)

DGGE profiles of sub-cultures from day 11 (designated S11 sub-cultures, Figure 4.1; Table 4.1) showed that five main phylotypes were enriched during 2MCPA dechlorination.

Sequencing of the bands labelled in Figure 4.1 confirmed that the DGGE profiles of the S11 sub-cultures were similar following the first sub-culture. For example, bands S11-1, S11-2, S11-3 (Figure 4.1) were common to all sub-cultures indicating that the communities were dominated by the same phylotypes in all S11 sub-cultures (see below). However, additional bands were observed at 10 mM 2MCPA (bands S11-4, Figure 4.1, Table 4.1), which represented phylotypes related to *Comamonas* (*Betaproteobacteria*), S11-7 related to *Azospirillum* (*Alphaproteobacteria*). A strain of *Comamonas* related to S11-4 was previously isolated from waste water that was able to degrade 2MCPA (Ji-Sook et al., 2003).

Bands S11-1, S11-2 and S11-3 (Figure 4.1) were present in both the 2 mM and 10 mM liquid sub-cultures and persisted through sub-culturing. A phylotype represented by band S11-1 (Figure 4.1, Table 4.1) was identical to one of the phylotypes enriched in the AS-RBT represented by band AS-6 (Figure 3.2, Table 3.1). However, a more dominant phylotype was represented by band S11-2 (Figure 4.1; Table 4.1) which persisted in subsequent sub-culturing alongside other strong bands (e.g. S11-3, Figure 4.1; Table 4.1). On the basis of 16S rRNA gene sequence analysis, S11-1 was closely related to *Caulobacter* (*Alphaproteobacteria*), band S11-2 (Figure 4.1, Table 4.1) was closely related to *Rhizobium* sp. (*Alphaproteobaceria*) (Nardi-Dei et al., 1999), and band S11-3 was closely related to *Acinetobacter radioresistens* (*Gammaproteobactia*). Strains of *Acinetobacter* are able to degrade some aliphatic chlorinated compounds; for example, isolates have been obtained from a waste water treatment plant in South Africa (Olaniran et al., 2005). A bacterium represented by S11-7 (Figure 4.1, Table 4.1) was closely related to *Azospirillum lipoferum* (*Alphaproteobacteria*).

All S11 sub-cultures showed only 50% dechlorination with both 2 mM and 10 mM 2MCPA, suggesting that the bacteria enriched might be producing dehalogenases able to act on only one isomer of DL-2MCPA.

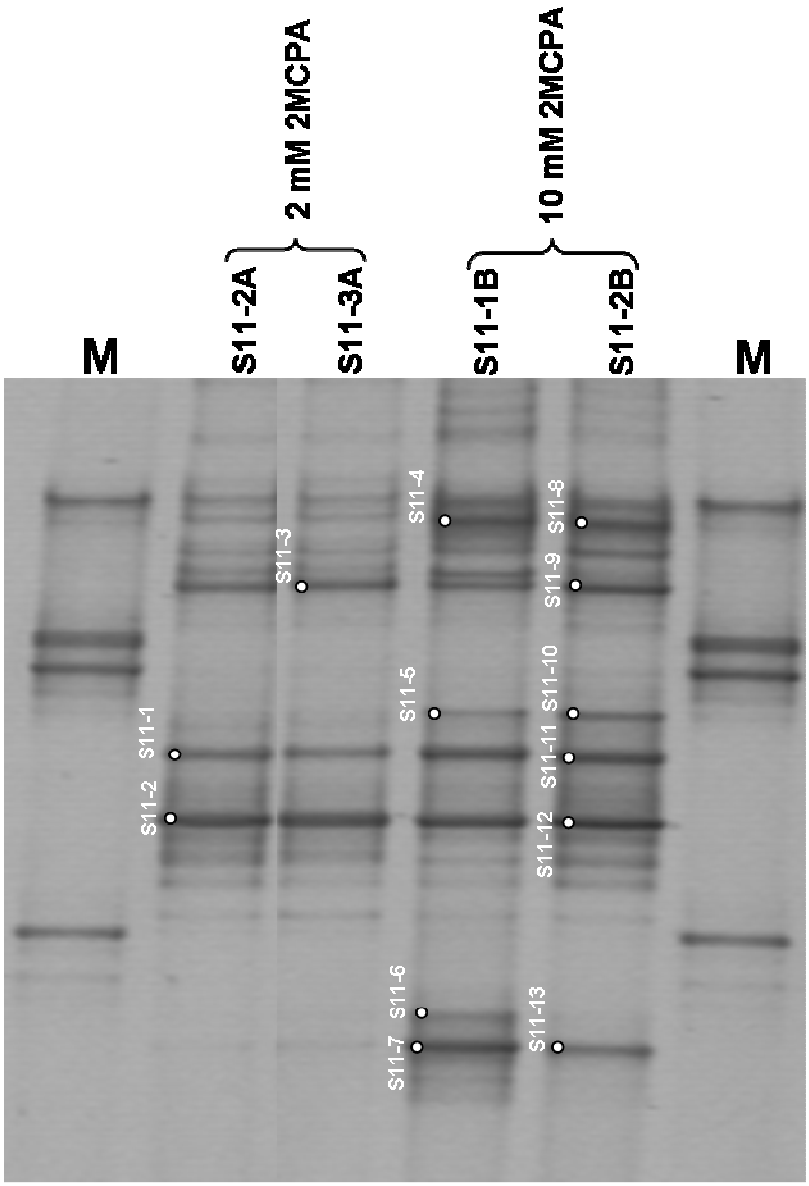

Figure 4.1 DGGE profile of bacterial 16S rRNA gene sequences obtained by PCR with DNA extracted from S11 sub-cultures in the presence of 2MCPA using 357FGC-518R primers. S11 numbers designations indicate bands excised for sequencing. All sub-cultures are designated using the format SX-YZ, where S = sub-culture enrichment; X = time of sampling (days) from AS-RBT; Y = number of sub-cultures; Z = 2MCPA concentration: either A (2 mM) or B (10 mM). M = marker.

Table 4.1 Closest sequence matches to excised DGGE bands of day 11 (S11) using BLASTN and the ribosome database project (RDP)

DGGE band ID[a,b]	Nearest match using NCBI nucleotide BLAST	Sequence similarity (%)	Sequence length (bp)	Phylogenetic affiliation	Nearest cultured relative using RDP	S-ab score[c] (%sequence similarity)
S11-1,11	*Caulobacter* sp. (EU857422)	100	169	*Alphaproteobacteria*	*Caulobacter fusiformis* (AJ007803)	1.000 (100)
S11-2, 12	Uncultured bacterium (EF409299)	98	169	*Alphaproteobacteria*	*Rhizobium* sp. (Y12351)	0.994 (98)
S11-3, 9	Uncultured bacterium (EU468036)	99	195	*Gammaproteobacteria*	*Acinetobacter radioresistens* (X81666)	0.968 (98)
S11-4, 8	*Comamonas* sp. (AJ550282)	97	194	*Betaproteobacteria*	*Comamonas* sp. (AJ550282)	0.917 (97)
S11-7, 13	Uncultured Rhodospirillaceae bacterium (EU426947)	100	169	*Alphaproteobacteria*	*Azospirillum lipoferum* (X79736)	1.000 (100)
S11-5,6,10	Poor sequence					

a. See Figure 4.1
b. Bands grouped together shared 100% sequence identity
c. As defined by Woese (1987)

4.3.1.2 DGGE profiles of the sub-cultures from day 16 (S16)

DGGE of the 16S rRNA gene sequences showed that eight main phylotypes were enriched in the S16 sub-cultures (Figure 4.2), which were assigned to *Alphaproteobacteria*, *Betaproteobacteria* and *Actinobacteria* (Table 4.2). The similarity in DGGE profiles (e.g. bands S16-1, S16-2, S16-6; Figure 4.2) which were common in all S16 sub-culture samples indicated that the communities were dominated by the same phylotypes (Figure 4.2, Table 4.2). However, additional bands were observed in 10 mM 2MCPA S16 sub-cultures (e.g., bands S16-12, S16-15; Figure 4.2).

The phylotype represented by S16-1 (Figure 4.2, Table 4.2) gave 97% sequence similarity with *Caulobacter* sp. (*Alphaproteobacteria*), and was one of the dominant organisms in the S16 sub-cultures. Band S16-2 (Figure 4.2, Table 4.2), showing 98% similarity with *Rhizobium* sp. (*Alphaproteobacteria*), was also a dominant S16 sub-culture member at 2 mM and 10 mM 2MCPA. Band S16-3 (Figure 4.2, Table 4.2) gave 96% sequence similarity with *Polaromonas* sp. (*Betaproteobacteria*). The phylotype represented by S16-6 (Figure 4.2, Table 4.2) showed similarity to *Azospirillum lipoferum* (*Alphaproteobacteria*) and, again, was present in all S16 sub-cultures, but appeared to increase in 2 mM 2MCPA sub-cultures and decrease in 10 mM 2MCPA sub-cultures.

Two bands which were observed at 2 mM but not at 10 mM 2MCPA (bands S16-4, S16-5; Figure 4.2), were closely related to *Bradyrhizobium* sp. (*Alphaproteobacteria*), and *Xanthobacter flavus* (*Alphaproteobacteria*). *Xanthobacter* spp. are known to degrade some chloroaliphatic compounds (Hill and Weightman, 2003; Ji-Sook et al., 2003; Marchesi and Weightman, 2003a), and a strain of *Xanthobacter flavus* isolated in South Korea was able to degrade DCA and 2MCPA (Song et al., 2004). *Xanthobacter autotrophicus* GJ10 has been reported to grow on αHAs (MCA, DCA, 2MCPA, 2MCBA (Janssen et al., 1985; Ploeg et al., 1995; Meusel& Rehm 1993).

The phylotype represented by band S16-12 (Figure 4.2, Table 4.2) persisted only in 10 mM 2MCPA S16 sub-cultures, and was assigned to *Rhizobium* sp. (*Alphaproteobacteria*). Band S16-15 (Figure 4.2, Table 4.2) was also enriched and pe-

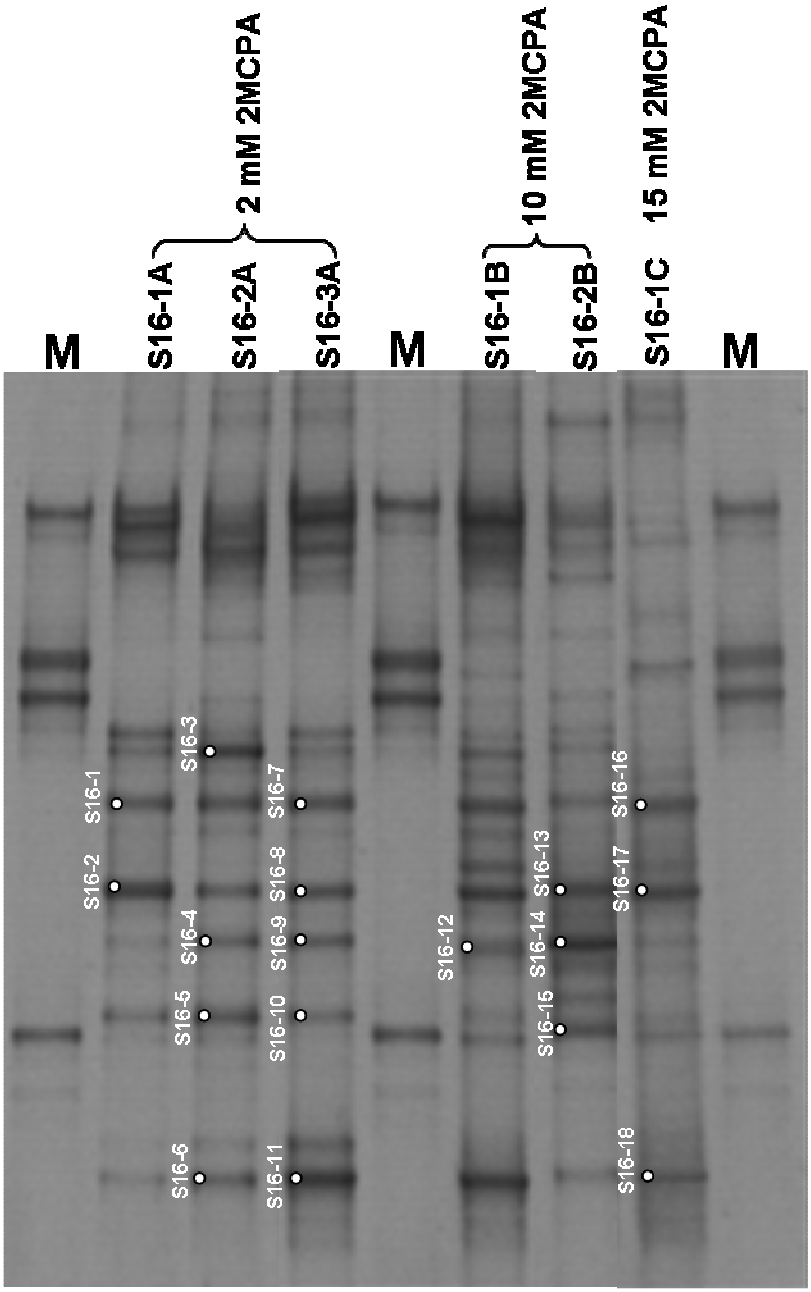

Figure 4.2 DGGE profile of bacterial 16S rRNA gene sequences obtained by PCR with DNA extracted from S16 sub-cultures in the presence of 2MCPA using 357FGC-518R primers. S16 numbers designations indicate bands excised for sequencing. All sub-cultures are designated using the format SX-YZ, where S = sub-culture enrichment; X = time of sampling (days) from AS-RBT; Y = number of sub-cultures; Z = 2MCPA concentration: either A (2 mM) or B (10 mM) or C (15 mM). M = marker.

Table 4.2 Closest sequence matches to excised DGGE bands of day 16 (S16) using BLASTN and the ribosome database project (RDP)

DGGE band ID[a,b]	Nearest match using NCBI nucleotide BLAST	Sequence similarity (%)	Sequence length (bp)	Phylogenetic affiliation	Nearest cultured relative using RDP	S-ab score[c] (%sequence similarity)
S16-1, 7,16	*Caulobacter* sp. (EU857422)	97	169	*Alphaproteobacteria*	*Caulobacter fusiformis* (AJ007803)	0.987 (98)
S16-2*, 8, 13, 17	Uncultured bacterium (AM697206)	98	170	*Alphaproteobacteria*	*Rhizobium* sp. (Y12351)	1.000 (100)
S16-3	Uncultured bacterium (EU622287)	96	196	*Betaproteobacteria*	*Polaromonas naphthalenivorans* (AY166684)	0.836 (85)
S16-4*, 9	*Bradyrhizobium* sp. (EU364719)	97	169	*Alphaproteobacteria*	*Bradyrhizobium genosp*. (Z94810)	1.000 (99)
S16-5, 10	*Azorhizobium caulinodans* (AP009384)	97	171	*Alphaproteobacteria*	*Xanthobacter flavus* (X94199)	1.000 (97)
S16-6, 11, 18	Uncultured Rhodospirillaceae bacterium (EU426947)	100	169	*Alphaproteobacteria*	*Azospirillum lipoferum* (X79736)	1.000 (100)
S16-12, 14	*Brucella abortus* (CP000888)	99	169	*Alphaproteobacteria*	*Rhizobium* sp. (AY691399)	1.000 (100)
S16-15	*Arthrobacter* sp. (EU833957)	98	173	*Actinobacteria*	*Arthrobacter nasiphocae* (AJ292364)	0.975 (98)

a. See Figure 4.2
b. Bands grouped together shared 100% sequence identity
c. As defined by Woese (1987)
* Isolated strain (see section 4.3.2.1)

rsisted through S16 sub-cultures at 10 mM 2MCPA and was closely related to *Arthrobacter* sp. (*Actinobacteria*).

4.3.1.3 DGGE profiles of the sub-cultures from day 20 (S20)

The 16S rRNA gene sequence analysis of the 2MCPA degrading bacteria of day 20 sub-cultures (S20, Figure 4.3) showed that six main phylotypes were enriched, five of which were *Alphaproteobacteria* (Table 4.3). The only phylotypes that were present in all sub-cultures (2 mM and 10 mM 2MCPA) were represented by bands S20-6, closely related to an *Azospirillum* sp. (Table 4.3) and, possibly, S20-1 (which did not give a good sequence) assuming this is the same DNA fragment as S20-9 which was identified as *Sphingobacteriaceae* phylotype (Table 4.3). The phylotype represented by band S20-3 (Figure 4.3; Table 4.3) persisted in the 2 mM 2MCPA S20 sub-cultures, and was identified as *Xanthobacter* sp. (*Alphaproteobacteria*). Band S20-4 (Figure 4.3, Table 4.3) represented a phylotype related to *Rhizobium* sp. (*Alphaproteobacteria*) that was enriched transiently at 2 mM 2MCPA, but the same phylotype represented by band S20-10 (Figure 4.3, Table 4.3) was one of the dominant bacteria in the 10 mM 2MCPA S20 sub-cultures. The phylotype represented by band S20-5 (Figure 4.3, Table 4.3) was closely related to *Bradyrhizobium* (*Alphaproteobacteria*) and was present only in 2 mM 2MCPA S20 sub-cultures. Band S20-11 (Figure 4.3, Table 4.3) represented a phylotype that persisted through sub-culturing in the 10 mM S20 sub-cultures, and was closely related to *Azospirillum* sp. (*Alphaproteobacteria*).

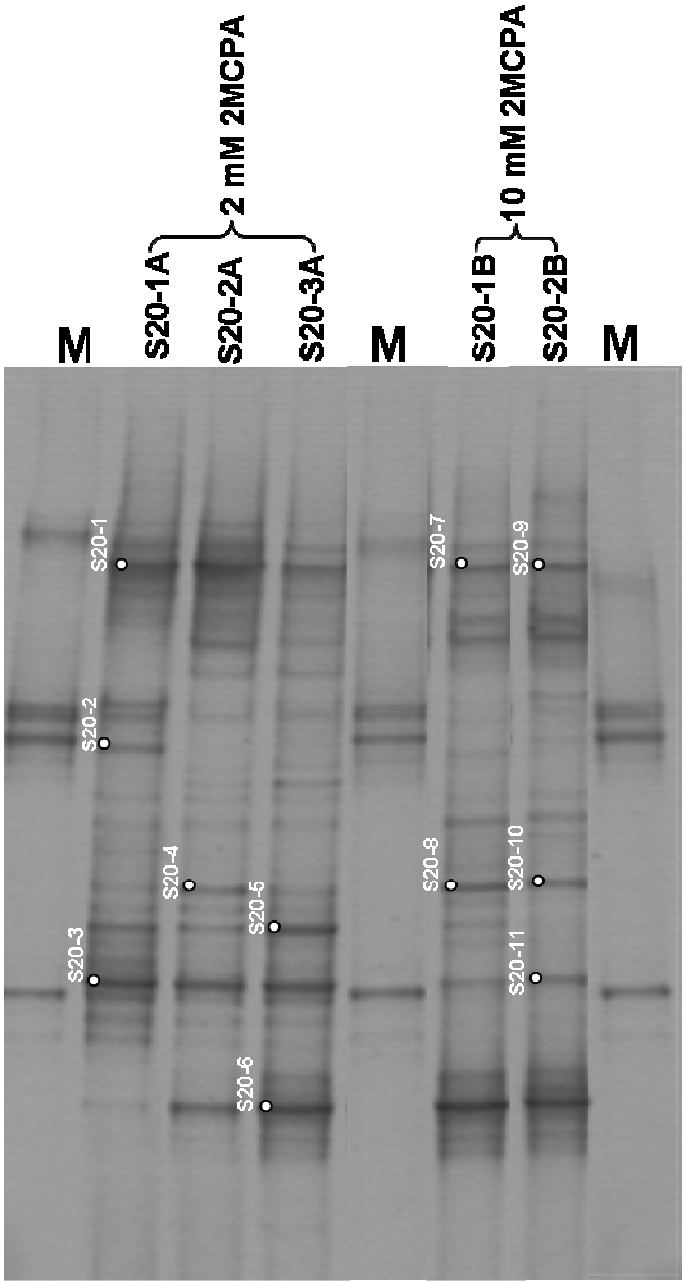

Figure 4.3 DGGE profile of bacterial 16S rRNA gene sequences obtained by PCR with DNA extracted from S20 sub-cultures in the presence of 2MCPA using 357FGC-518R primers. S20 numbers designations indicate bands excised for sequencing. All sub-cultures are designated using the format SX-YZ, where S = sub-culture enrichment; X = time of sampling (days) from AS-RBT; Y = number of sub-cultures; Z = 2MCPA concentration: either A (2 mM) or B (10 mM). M = marker.

Table 4.3 Closest sequence matches to excised DGGE bands of day 20 (S20) using BLASTN and the ribosome database project (RDP)

DGGE band ID[a,b]	Nearest match using NCBI nucleotide BLAST	Sequence similarity (%)	Sequence length (bp)	Phylogenetic affiliation	Nearest cultured relative using RDP	S-ab score[c] (%sequence similarity)
S20-1, 7, 9	Uncultured *Sphingobacteriaceae bacterium* (EF695533)	96	189	*Bacteroidetes*	*Sphingobacteriaceae bacterium* (EU057831)	0.973 (95)
S20-3	Uncultured alphaproteobacterium (EF699691)	99	170	*Alphaproteobacteria*	*Xanthobacter* sp. (AJ514456)	1.000 (99)
S20-4, 8,10	Uncultured bacterium (AM697206)	98	169	*Alphaproteobacteria*	*Rhizobium* sp. (AY064413)	0.994 (100)
S20-5*	*Bradyrhizobium* sp. (EU364719)	99	170	*Alphaproteobacteria*	*Bradyrhizobium genosp* (Z94810)	1.000 (99)
S20-6	Uncultured bacterium (AB307662)	99	170	*Alphaproteobacteria*	*Azospirillum* sp. (AB049110)	0.975 (99)
S20-11	Uncultured bacterium (AB307664)	98	169	*Alphaproteobacteria*	*Azospirillum* sp. (AJ864460)	0.968 (98)
S20-2	Poor sequence					

a. See Figure 4.3
b. Bands grouped together shared 100% sequence identity
c. as defined by Woese (1987)
* Isolated strain (see section 4.3.2.1)

4.3.2 Isolation of 2MCPA-utilizing bacteria from the RBTs

4.3.2.1 Isolation of 2MCPA-utilizing bacteria from AS-RBT sub-cultures

Two pure cultures, one identified as *Bradyrhizobiaceae/"Afipia"* sp. KH3 and the other as *Rhizobium* sp. KH31, were isolated from the AS-RBT sub-cultures (S16 and S20) described in section 4.3.1 (Figure 4.4, Table 4.4). The strain *Bradyrhizobiaceae/"Afipia"* sp. KH3 was obtained from the 10 mM 2MCPA S16 and S20 sub-cultures, but also represented a dominant population in the 2 mM 2MCPA S16 and S20 sub-cultures. Both strains, KH3 and KH31, were seen as dominant phylotypes in the DGGE profiles of sub-cultures from which they were isolated (Figure 4.4), but no representative of these two isolates were observed in the original AS-RBT (Figure 4.4).

Random amplified polymorphic DNA (RAPD; see section 2.6.4) analysis of *Bradyrhizobiaceae/"Afipia"* isolates from different sub-cultures showed identical RAPD profile (result not shown), and therefore these isolates were considered to belong to the same species (Rivas et al., 2004) and strain KH3 was chosen for further analysis.

4.3.2.2 Isolation of 2MCPA-utilizing bacteria from soil RBTs

The same experimental approach as described in section 4.3.1 was also carried out to isolate 2MCPA-degrading bacteria from the soil RBTs (sections 3.3.3 and 3.3.4), but not with extensive DGGE profile monitoring, and only at 10 mM 2MCPA (see section 2.4.1). Two pure cultures, designated strains *Herbaspirillum* sp. KH17 and *Methylobacterium* sp. KH4, were isolated from HS-RBT and LS-RBT, respectively (Table 4.4). Strain KH17 corresponded to a phylotype presented in the original HS-RBT (see Figure 4.5), and a very faint band in the DGGE profile of the LS-RBT (day 37), which was not sequenced seen in the LS-RBT DGGE profile, had the same electrophoretic mobility as strain KH4 which was isolated from the LS-RBT (Figure 4.5).

Several *Herbaspirillum* isolates were purified on SBS 2MCPA agar, and showed the same RAPD profile (data not shown). Therefore, they were considered to belong to

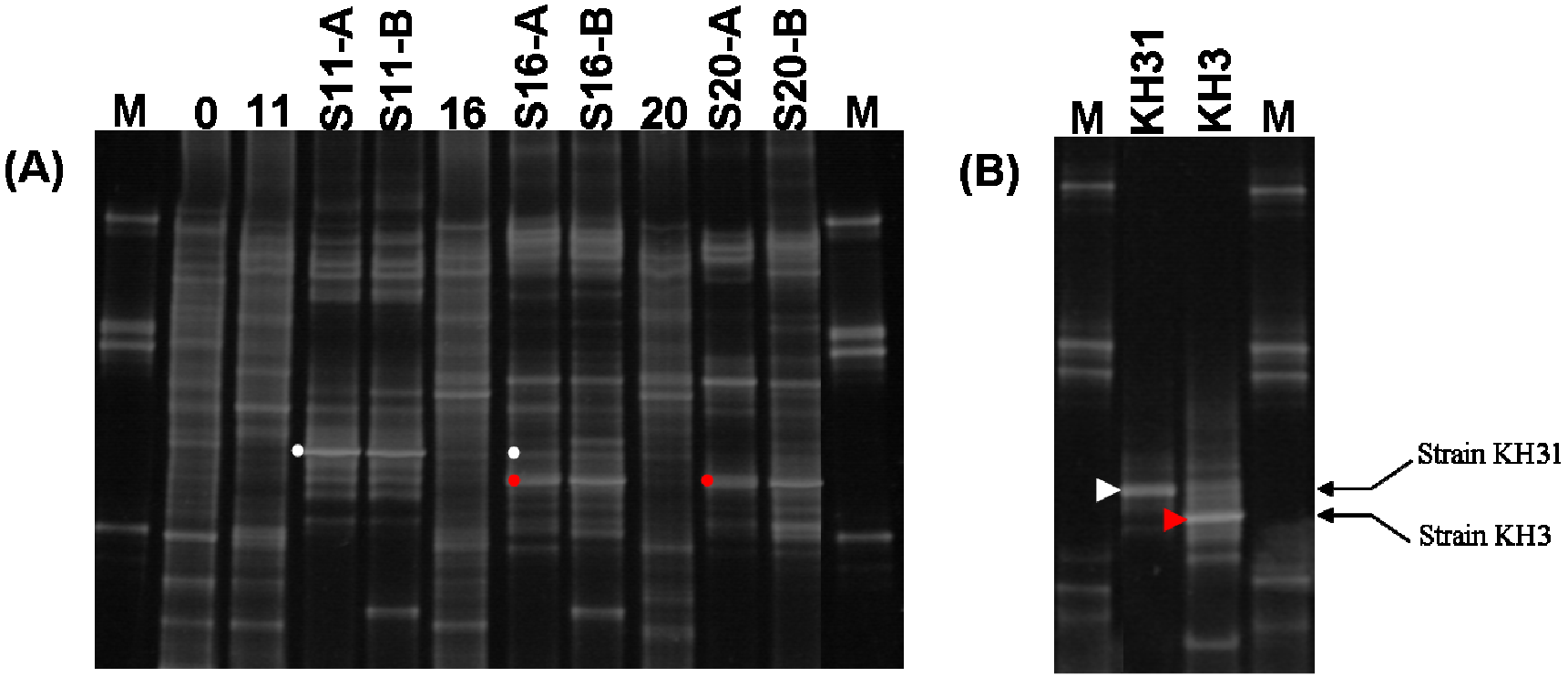

Figure 4.4 DGGE profile of bacterial 16S rRNA gene sequences obtained by PCR using 357FGC-518R primers from AS-RBT, liquid sub-cultures of plate wash of the AS-RBT (A) and pure culture (B). Numbers above each lane 0, 11, 16, 20 represent the day of sampling from AS-RBT (see Figure 3.1). S11, S16, S20 designations indicate sub-cultures of day 11, 16 and 20. All sub-cultures designated the format SX-Z, where S = sub-culture enrichment; X = time of sampling (days) from AS-RBT; Z = 2MCPA concentration: either A (2 mM) or B (10 mM). KH3, *Afipia* sp. strain KH3 indicated by red arrow. KH31, *Rhizobium* sp. strain KH31 indicated by white arrow. Circles represent the phylotypes enriched in the sub-cultures which gave identical 16S rRNA gene sequence to the isolated strains shown by arrows (given the same colour). M = marker.

Table 4.4 Phylogenetic affiliation and closest match of 16S rRNA gene sequences obtained from 2MCPA-utilizing isolates.

Strain	Closest match using NCBI	Sequence similarity (%)	Alignment length (bp)	Phylogenetic affiliation	Closest match using RDP
Afipia sp. strain KH3	*Oligotropha carboxidovorans* (AB099660)	98	919	*Alphaproteobacteria*	*Afipia* sp. 4LS2 (FJ851429)
Rhizobium sp. strain KH31	Uncultured bacterium (AM697206)	100	830	*Alphaproteobacteria*	*Rhizobium* sp. DCP2 (AY064412)
Herbaspirillum sp. strain KH17	Uncultured bacterium (DQ532247)	99	862	*Betaproteobacteria*	*Herbaspirillum* sp. DA1 (AJ430686)
Methylobacterium sp. strain KH4	*Methylobacterium* sp. (AM910536)	99	849	*Alphaproteobacteria*	*Methylobacterium* sp. (DQ512770)

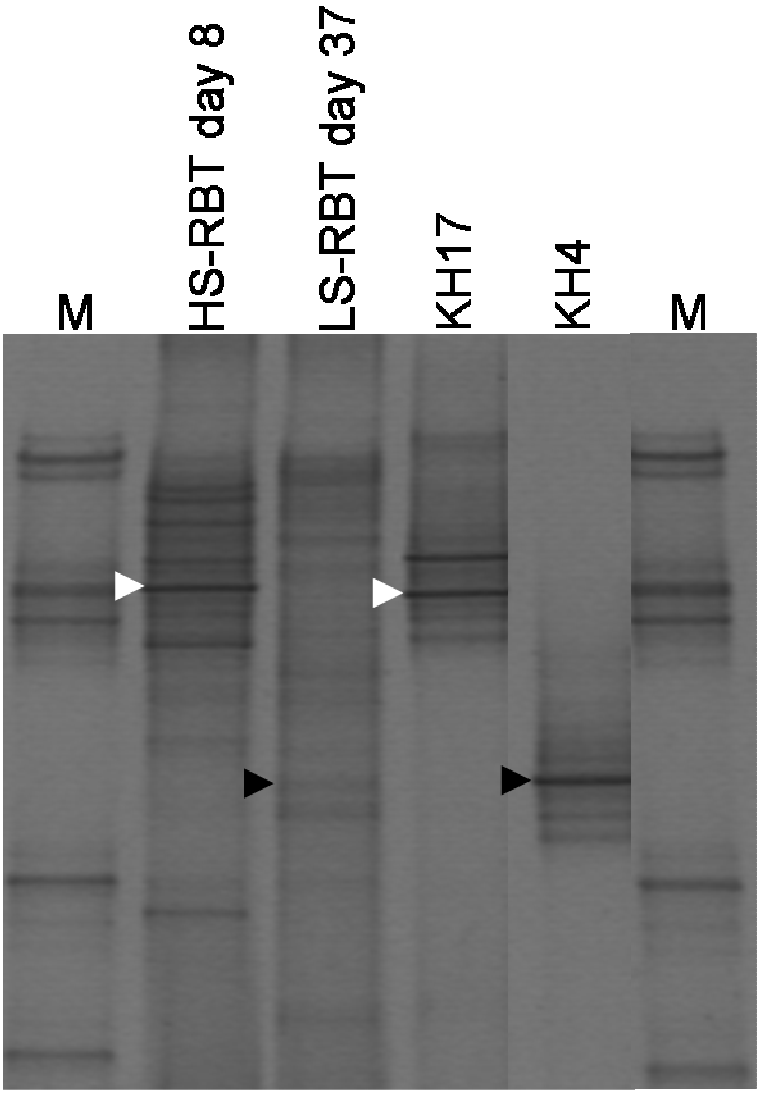

Figure 4.5 DGGE gel showing diversity of 16S rRNA gene fragments amplified DNA extracted from soil RBTs and pure cultures. Lane labelled HS-RBT day 8 indicates DGGE pattern of day 8 from HS-RBT refer to Figure 3.4A, lane labelled LS-RBT day 37 DGGE pattern of day 37 from LS-RBT refer to figure 3.4B. KH17 represent the strain isolated from HS-RBT, *Herbaspirillum* sp., indicated by white arrow (the isolates has two copies of 16S rRNA gene). KH4 represent the strain isolated from LS-RBT, *Methylobacterium* sp., indicated by black arrow. M = marker.

the same species (Rivas et al., 2004) and only strain KH17 was chosen as a representative for further analysis.

4.3.3 Phylogenetic affiliations of the isolated strains

Phylogenetic analysis of the 16S rRNA gene sequences was used to determine the closest relatives of the isolated strains, and all were assigned to genera in the class *Alphaproteobacteria*. Strain KH3, isolated from the sub-cultures containing 10 mM 2MCPA, represented a dominant population enriched in the sub-cultures of day 16 (S16-4, Figure 4.2; Table 4.2) and day 20 (S20-5, Figure 4.3; Table 4.3). Phylogenetic analysis showed that, this organism was closely related to *Oligotropha carboxidovorans* (Malik et al., 2003), *Afipia* (Green et al., 2010), and *Bradyrhizobium* (Marchesi and Weightman, 2003a) (*Alphaproteobacteria*), but was assigned to *Afipia* sp. on the basis of the tree shown in Figure 4.6. It has been reported that these genera are very closely related based on 16S rRNA gene sequences (Islam et al., 2008; Willems 2001, 1992; Noisangiam 2010).

Amplification and sequencing of the DNA gyrase subunit B gene (*gyrB*) also indicated that strain KH3 was most closely related to *Bradyrhizobium* sp (79% sequence similarity) and *Oligotropha carboxivorans* OM5 (70% sequence similarity) (results not shown). Strain KH31, also isolated from AS-RBT sub-cultures, and was identified as a *Rhizobium* sp. The *Rhizobium* sp. strain KH31 contains an insert sequence in its 16S rRNA gene sequence that was not similar to any 16S from data base and gave similarity to a species of *Rhizobium* isolated from soil in China (Ren et al., 2010).

The strains isolated from soil-RBTs sub-cultures were beta and alphaproteobacterial genera (Figure 4.6). Strain KH17 was assigned to the family *Oxalobacteriaceae* (Fig 4.6) and the genus *Herbaspirillum*. Strain KH4 was assigned to *Methylobacterium* (99% sequence identity; Figure 4.6).

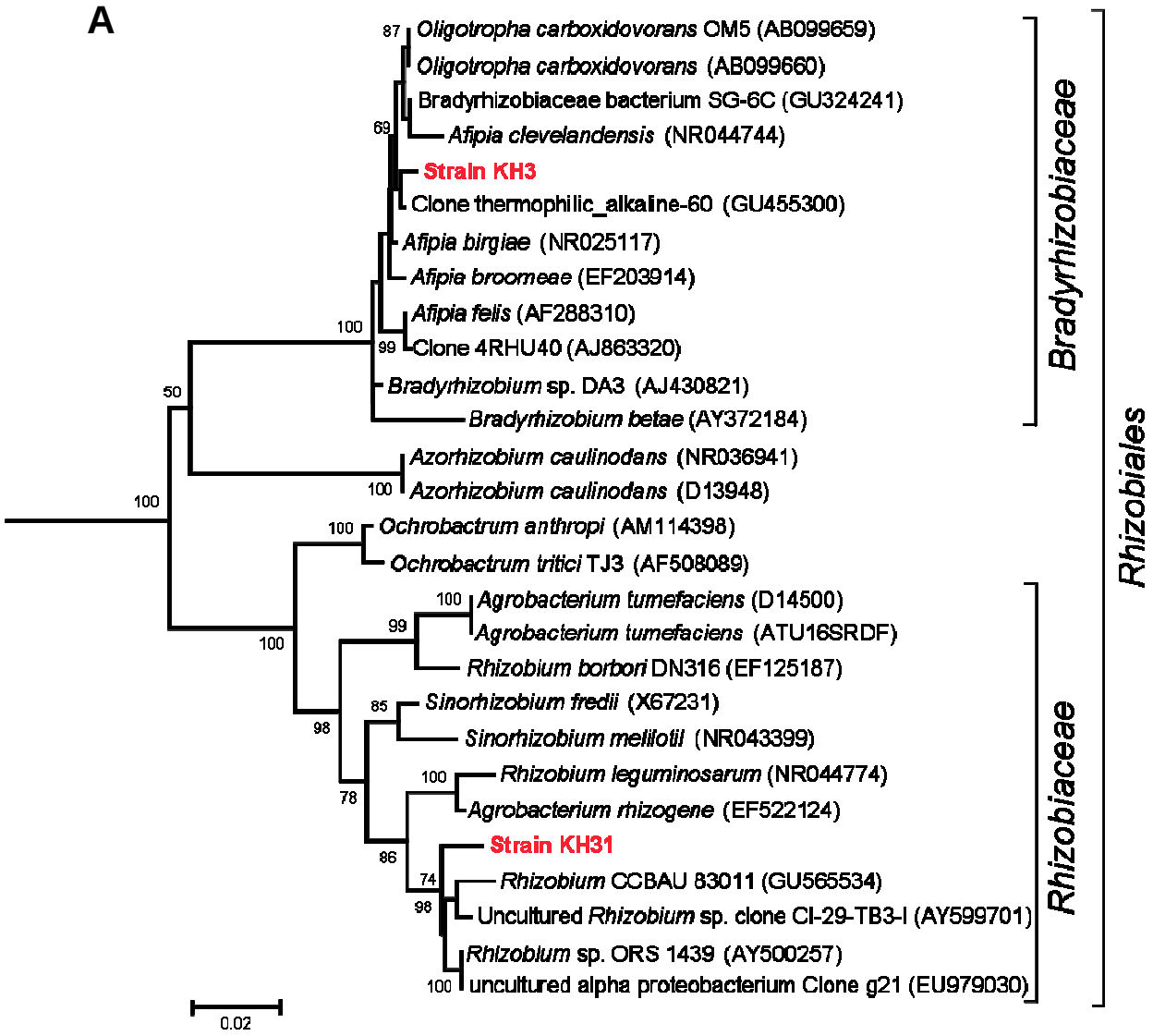

Figure 4.6 For legend, see next page.

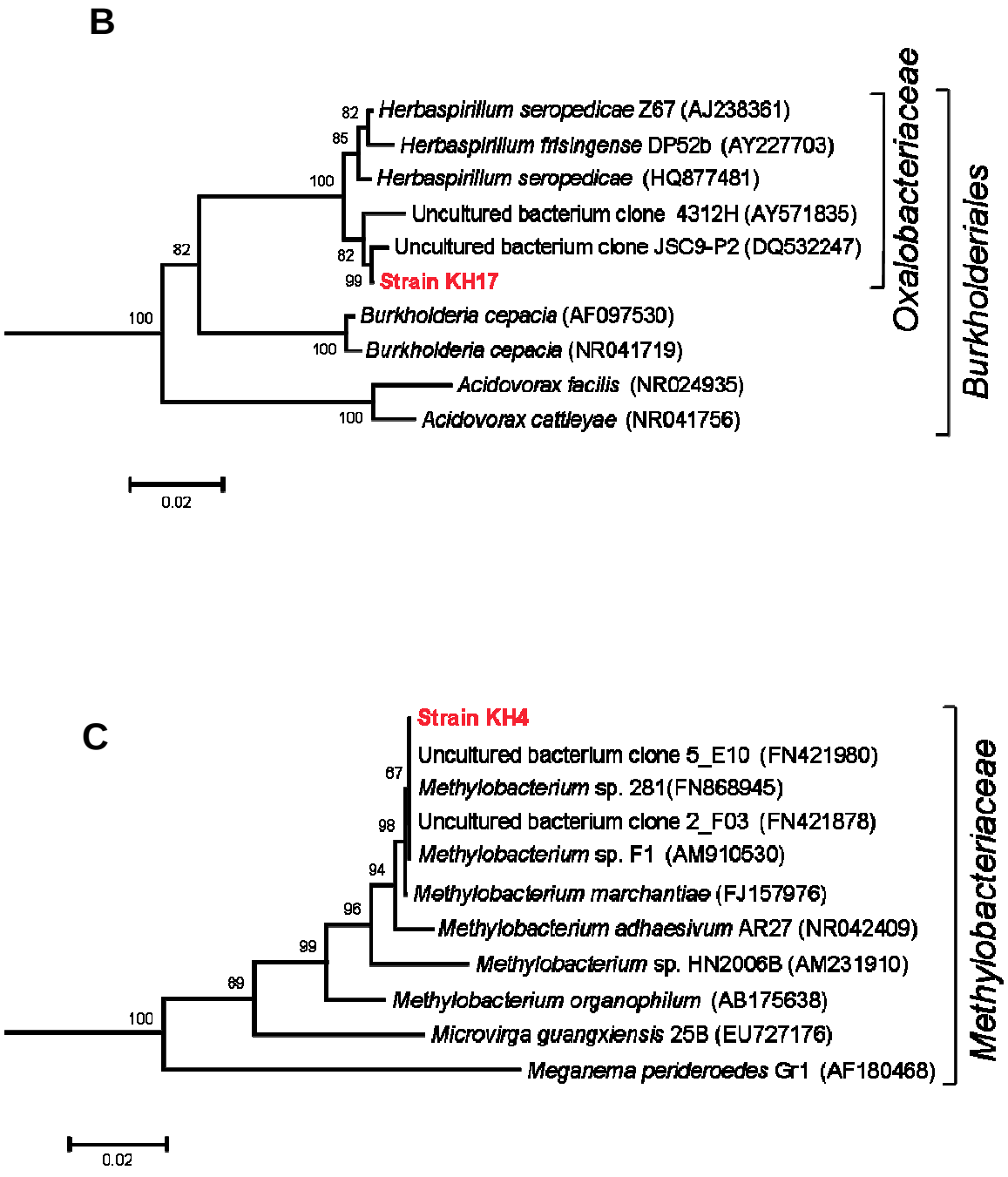

Figure 4.6 Dendograms illustrating the phylogenetic relationships between the isolated strains (coloured red), and references from the database based on alignment of the 16S rRNA gene. A) the phylogenetic relationships between strains KH3, KH31 and other strains from database, based on alignment of 950 nucleotides from the 16S rRNA gene, the out group was (*Herbaspirillum*; AY227703, *Burkholderia*; NR041719, *Acidivorax*; NR041756). B) the phylogenetic relationships between strains KH17 and other strains from database, based on alignment of 900 nucleotides from the 16S rRNA gene, the out group was (*Rhizobium*; AY500257, *Rhizobium*; EF125187, *Bradyrhizobium*; AY372184). C) the phylogenetic relationships between strains KH4 and other strains from database, based on alignment of 900 nucleotides from the 16S rRNA gene, the out group was (*Herbaspirillum*; AY227703, *Burkholderia*; NR041719, *Acidivorax*; NR041756). All trees constructed using the Neighbor-Joining method. Boot strap values >50%, based on 1000 replicates, are shown as a percentage at the nodes. Scale bar represents the number of base substitutions per site.

4.3.4 Growth of the isolated strains on solid media

Afipia sp. strain KH3 grew on 10 mM 2MCPA solid medium, producing 2-3 mm diameter colonies, white creamy, circular, entire and smooth. The strain also grew on R2A, but no growth observed on YEM medium or nutrient agar (Table 4.5) (Ohta and Hattori, 1980; Müller, 1995; Moosvi et al., 2005). It has been reported that nutrient broth and yeast mannitol agar have inhibition effect on the growth of *Afipia* species (inhibition of *Afipia* species by NaCl; Müller, 1995) (Moosvi et al., 2005). However, members of *Bradyrhizobium* species have been reported to grow on YEM medium (Holben et al., 1988).

Rhizobium sp. strain KH31 produced white creamy colonies, 2 mm in diameter, on 10 mM 2MCPA medium. Colonies of *Herbaspirillum* sp. strain KH17 on SBS 2MCPA (10 mM) agar were creamy white with a diameter of 3-4 mm, circular, smooth, convex and tough, recognized by formation of typical pellicle (clump) (Kirchhof et al., 1997; Schmid et al., 2006). Strain KH17 grew well on cultivation media, such as nutrient agar, YEM (Schmid et al., 2006) and R2A (Table 4.5).

Strain *Methylobacterium* KH4 produced pink colonies on 2MCPA plates, but gave cohesive mat when grown in liquid medium (SBS 2MCPA) (La Roche and Leisinger, 1991). Colonies of strain KH4 were 1-2 mm in diameter, circular, convex, had a smooth surface, with an entire margin. This organism also grew on nutrient agar, R2A and YEM (Table 4.5).

4.3.5 Growth of the isolated strains on different carbon sources

The range of substrates (carbon source) on which the isolated strains showed growth is summarised in Table 4.6. In addition to 2MCPA, *Afipia* sp. strain KH3 utilized DCA, 22DCPA, acetate and succinate as growth substrates, but no growth was observed with MCA, TCA, 2MCBA or glucose (Table 4.6). Strains of *Afipia* sp. isolated on DCA contained *dehI* and *dehII*, were able to degrade TCA (Zhang et al., 2009b). Moosvi et al. (2005) reported that strains of *Afipia felis* isolated from Antarctica were able to grow on acetate and succinate but not glucose. The *Herbaspirillum* sp. strain KH17 was able to utilize 2MCPA, MCA, DCA, 22DCPA, sodium acetate and sodium succinate, but not TCA, 2MCBA or glucose. This is consistent with a strain of *Herbaspirillum* sp. isolated from well water, which was not

Table 4.5 Ability of the strains to grow on complex media

Strain	Growth on solid media[a]		
	NA[b]	R2A[b]	YEM[b]
Afipia sp. strain KH3	-	+	-
Herbaspirillum sp. strain KH17	+	+	+
Methylobacterium sp. strain KH4	+	+	+

a. -, No growth, +, growth was detected

b. NA nutrient agar, R2A agar, YEM, yeast extract mannitol agar

Table 4.6 Substrate specificity of the isolated strains

Substrate[a]	Growth[b]		
	***Afipia* sp. strain KH3**	***Herbaspirillum* sp. strain KH17**	***Methylobacterium* sp. strain KH4**
Glucose	-	-	+
Acetate	+ +	+ +*	+ +
Succinate	+ +	+ + +*	+ +
MCA	-	+	-
DCA	+ +	+ +	-
TCA	-	-	-
2MCPA	+ +	+ +*	+ +
22DCPA	+ +	+ + +*	-
2MCBA	-	-	-

a. Carbon source were added at 5 mM 2MCPA in SBS medium (see section 2.1.2). MCA; monochloroacetic acid, DCA; dichloroacetic acid, TCA; trichloroacetic acid, 2MCPA; 2-chloropropionic acid, 22DCPA; 2,2-dichloropropionic acid, 2MCBA; 2-chlorobutric acid
b. -,+, ++, +++, estimate for relative growth/ activity based on turbidity and [Cl^-]
* Aggregate formation observed

able to utilize glucose (Ding and Yokota, 2004). Strain *Methylobacterium* sp. KH4 utilized 2MCPA as growth substrate, but showed no growth with any other halogenated substrate tested (Table 4.6). *Methylobacterium* sp. strain HJ1 isolated previously on 22DCPA was also not able to grow on MCA and DCA, or glucose (Jing and Huyop, 2008).

Repeated attempts to grow strain *Rhizobium* KH31 from freezer stock failed, despite its original isolation and growth on 2MCPA, and its dehalogenase activity in cell-free extracts (see section 4.3.8).

4.3.6 Growth rates on 2MCPA of the bacterial strains isolated from the RBTs

The growth rates of the organisms on defined mineral medium with 10 mM 2MCPA as sole carbon and energy source were determined in liquid media. *Afipia* sp. strain KH3 gave a specific growth rate 0.036 (± 0.003) h^{-1} (Figure 4.7A, Table 4.7), and *Herbaspirillum* sp. strain KH17 had a specific rate of 0.11 (± 0.01) h^{-1} (Figure 4.7B, Table 4.7). *Methylobacterium* sp. strain KH4 grew with a specific growth rate of 0.0020 (± 0.0003) h^{-1} (Figure 4.7C, Table 4.7), using chloride release as a proxy for growth (i.e. data from chloride release was used to determine growth rate). It has been reported previously that growth rate of bacterial species which are able to degrade halogenated compounds reflect their rates of dechlorination (Kim and Rhee, 1997).

Complete dehalogenation of 10 mM 2MCPA by strain KH3 was achieved in ~9 days of incubation after a lag phase of about 60 h, and doubling time of 19 h (Figure 4.7A). Strain KH17 degraded all the 2MCPA after 2 days of incubation with a lag phase of about 20 h and doubling time of 6 h (Figure 4.7B). Complete dehalogenation of 10 mM of 2MCPA by strain KH4 was achieved after 39 days of incubation. A lag phase of about 70 h was observed prior to the 2MCPA biodegradation and the doubling time of 345 h (Figure 4.7C). Growth rate was not determined for *Rhizobium* sp. strain KH31 as repeated attempts to grow the organism from freezer stock failed.

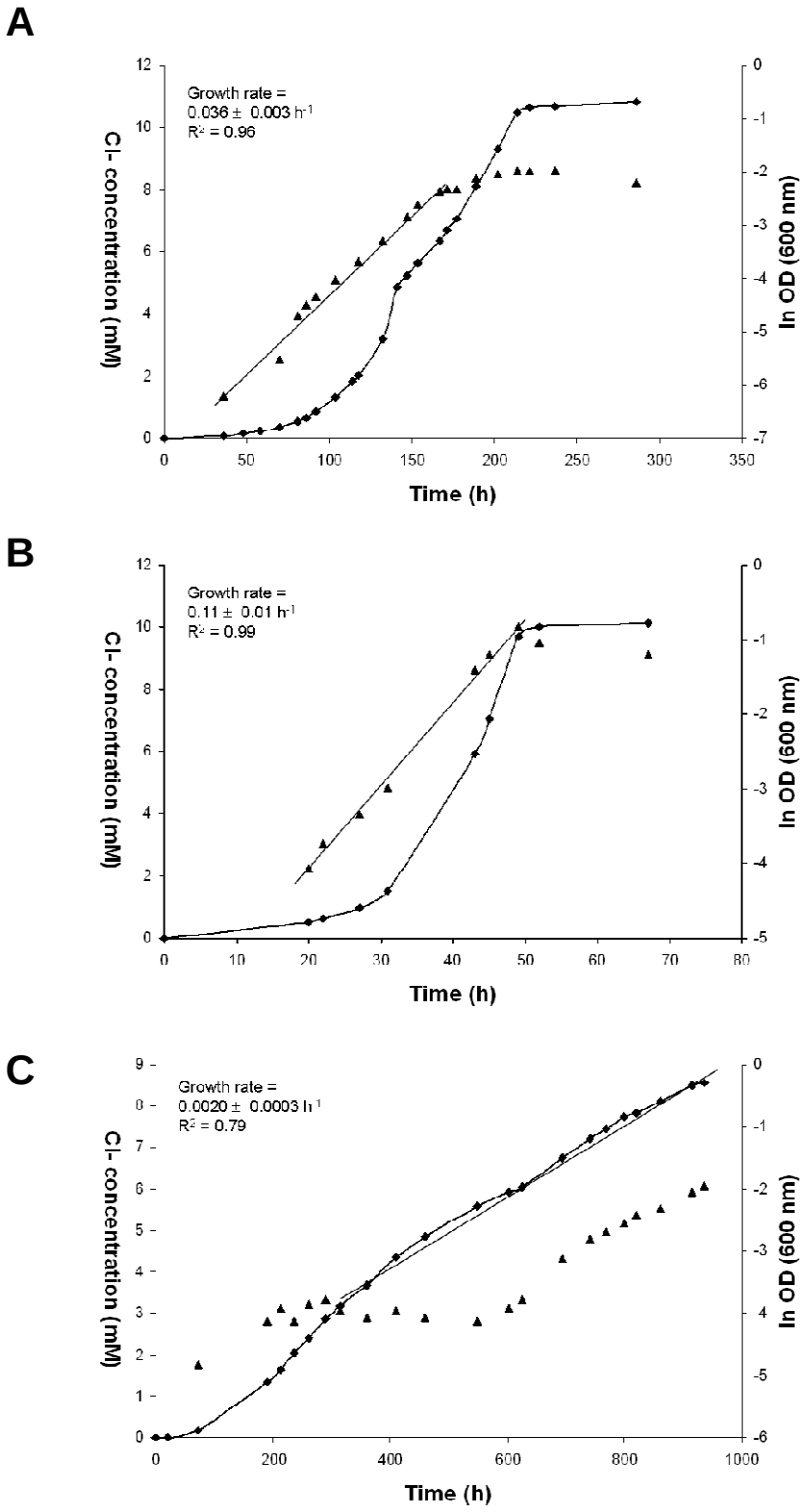

Figure 4.7 Growth and dechlorination curves for *Afipia* sp. strain KH3 (A), *Herbaspirillum* sp. strain KH17 (B), and *Methylobacterium* sp. KH4 (C) grown on 10 mM SBS 2MCPA at 20°C. Absorbance at 600 nm (▲), Cl^- release (mM) (•). R^2 = linear regression proportion of variability of data.

Table 4.7 Relative dehalogenase activity in the cell-free extracts (CFEs) and other characteristies of the isolated strains

Bacterial strain	Growth rate[a] (h^{-1})	Dehalogenase specific activity (U/ mg protein)[b,c]					Gel zymography[d]		deh PCR[e]		
		MCA	DCA	2MCPA	22DCPA	2MCBA	MCA	2MCPA	dehI[f]	dehI[g]	dehII[h]
Afipia sp. KH3	0.036 (±0.003)	0.06 (100)	0.06 (93)	0.02 (31)	0.05 (73)	ND	1	1	+	-	+
Rhizobium sp. KH31	ND	0.04 (100)	0.02 (44)	0.01 (22)	0.01 (38)	0.01 (33)	ND	ND	+	-	+
Herbaspirillum sp. KH17	0.11 (± 0.01)	0.29 (100)	0.03 (11)	0.36 (216)	0.09 (31)	0.16 (55)	1	1	+	+	-
Methylobacterium sp. KH4	~ 0.0020 (±0.0003)	0.21 (100)	0.10 (46)	0.26 (123)	0.17 (78)	ND	2	2	-	-	-

a. Average value based on three determinations (data represent mean ± SD of three independent experiments)

b. A unit of dehalogenase activity is defined as the amount of enzyme required to convert 1 µmol substrate / min

c. Values in parentheses represent percentage activity relative to MCA. MCA; monochloroacetic acid, DCA; dichloroacetic acid, 2MCPA; 2-chloropropionic acid, 22DCPA; 2,2-dichloropropionic acid, 2MCBA; 2-chlorobutric acid

d. Number of band stained with MCA or 2MCPA (section 4.3.8, Figure 4.10)

e. PCR amplification of *dehI* and *dehII* genes (Hill et al., 1999); -, No PCR product, +, positive PCR product

f. Short *dehI* gene (230 bp) using $dehI_{for1}$/$dehI_{rev1}$ primer sets described by Hill et. al., (1999; section 2.6.2)

g. Long *dehI* gene (504 bp) using $dehI_{for1}$/$dehI_{rev2}$ primer sets described by Hill et. al., (1999; section 2.6.2)

h. *dehII* gene (422 bp) using $dehII_{for1}$/$dehll_{rev1}$ primer sets described by Hill et. al., (1999; section 2.6.2)

ND, Not determined

4.3.7 Presence of *dehI* and *dehII* genes in the isolated strains

All four isolated strains were screened by PCR for the presence of *dehI* and *dehII* genes (Figure 4.8). Both *dehI* (short and long *deh* primer sets; see section 2.6.2) and *dehII* PCR products were obtained using strain *Afipia* KH3 DNA, but the product obtained using the long primer pair was faint (Figure 4.8). Strain *Rhizobium* KH31 gave PCR product with *dehI* (short) and *dehII*. No *dehII* PCR product was detected using DNA from *Herbaspirillum* sp. strain KH17, but *dehI* (short and long *deh* primer) PCR products were obtained from this strain (Figure 4.8). No PCR products of the correct size were obtained from strain *Methylobacterium* KH4 using short *dehI*, long *dehI* primer set (data not shown) and *dehII* gene primers (Figure 4.8).

The shorter *dehI* PCR product (230 bp) obtained from strain KH17 with $dehI_{for1}$/$dehI_{rev1}$ was used for cloning and sequencing. Based on data obtained strain KH17 do not have group II *deh* gene (Figure 4.8). These finding suggest that *Herbaspirillum* sp. strain KH17 may only have a group I dehalogenase which acts on DL-2MCPA.

PCR product of *dehI* from strain *Herbaspirillum* KH17, was cloned and numbers of *dehI* recombinants were sequenced to determine their relationship with previously characterized dehalogenases (see section 2.14). Figure 4.9 shows a phylogenetic tree illustrating the relationship between the *deh* genes sequence isolated from this bacteria and the other group I *deh* genes previously reported in the literature.

The phylogenetic tree constructed by Neighbor-joining method showed that that the group I dehalogenase from strain KH17 was identical at the nucleotide level to *dehI* genes gene from *Herbaspirillum* DA1 (*Betaproteobacteria*) and was assigned to sub-group B (Marchesi and Weightman, 2003a) which also contain DL-$_{DEX}$ (Figure 4.9) and other DL-2MCPA specific dehalogenaes (Hill et al., 1999).

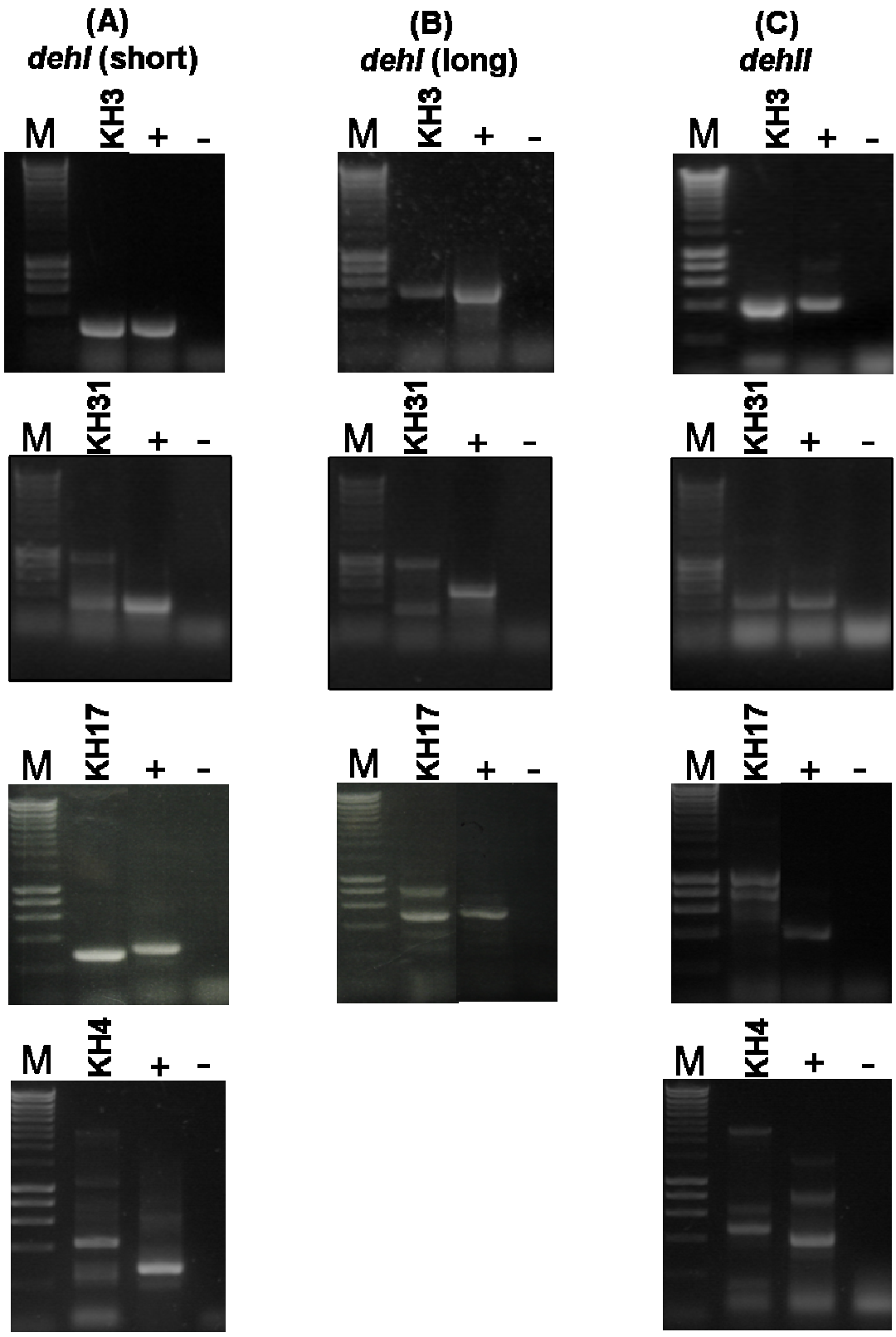

Figure 4.8 Agarose gel showing PCR products from the isolated strains using group I (*dehI)* and group II (*dehII*) dehalogenase primers. (A) *dehI* (short) gene PCR. (B) *dehI* (long) gene PCR. (C) *dehII* gene PCR. KH3, *Afipia* sp.; KH31, *Rhizobium* sp.; KH17, *Herbaspirillum* sp.; KH4, *Methylobacterium* sp. M = marker; + = positive PCR control; - = negative PCR control.

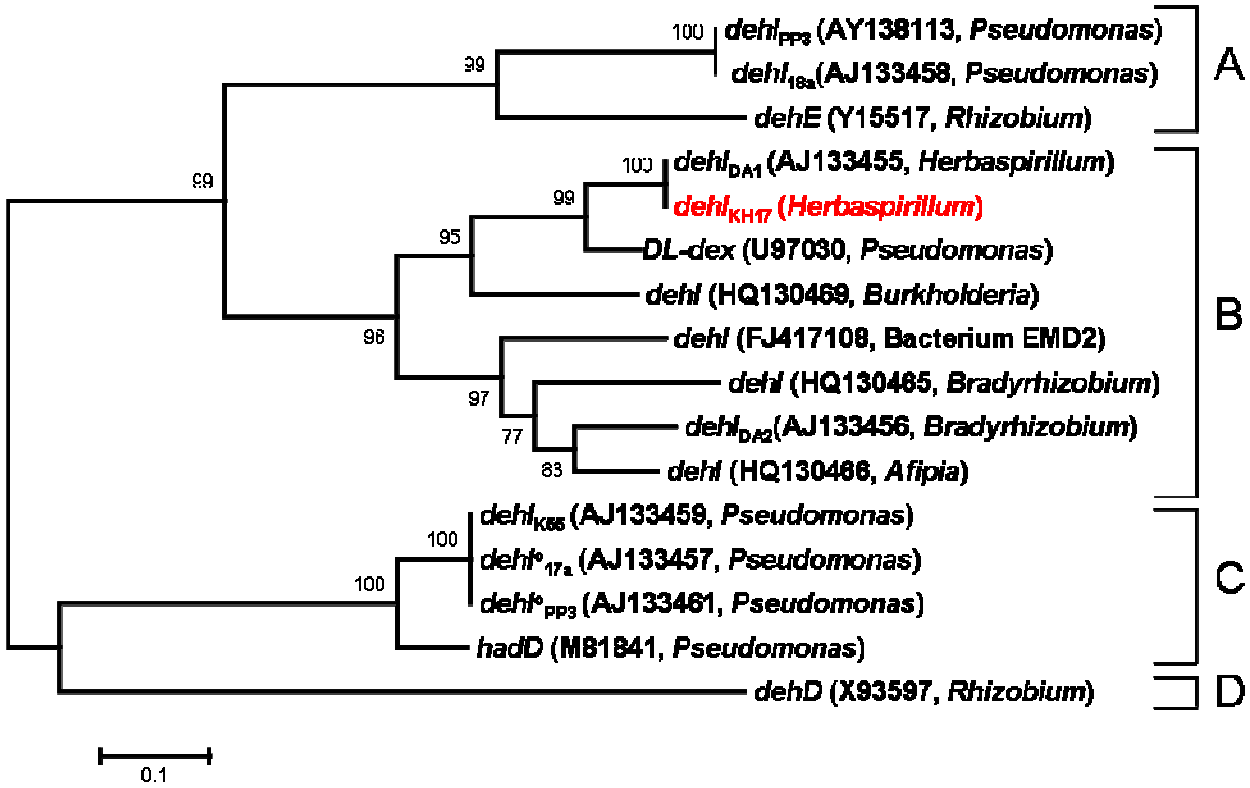

Figure 4.9 Dendogram illustrating the phylogenetic relationship between dehalogenase genes (*dehI*) amplified from *Herbaspirillum* sp. strain KH17 in this study (coloured red) and references from the database: based on alignment of 229 nucleotides. The tree was constructed using the Neighbor Joining method and the Jukes-Cantor algorithm. Boot strap values > 50%, based on 1000 replicates are shown as a percentage at the nodes. The scale bar represents the number of base substitutions per site.

4.3.8 Dehalogenase activity in cell-free extracts (CFE) from the isolated strains

Cell free extracts (CFE) were prepared from cultures of the isolates grown on SBS acetate + 2MCPA, and were tested for activity with a range of αHAs (Table 4.7). In strains KH3 and KH31 the highest specific activity was with MCA, whereas in strains KH17 and KH4 2MCPA gave the highest specific activity, but the specific activities for strains KH17 and KH4 with MCA were 3-5 fold higher than for strain KH3. Relative activities were MCA>DCA>22DCPA>2MCPA for strains KH3 and KH31, but 2MCPA>MCA>22DCPA>DCA for strains KH17 and KH4. Dehalogenase specific activity of strain KH31 with all αHA substrates, was very low compared to the other three isolates.

Significant activity was detected within the cell debris (pellet; see section 2.11) removed from the crude cell extracts when assayed with MCA and 2MCPA (Table 4.8). The pellet from *Afipia* sp. strain KH3 showed specific activities 3-fold higher than that in CFE, but *Rhizobium* sp. strain KH31 gave specific activities about the same specific activity as the CFE for both MCA and 2MCPA. The pellet from *Herbaspirillum* sp. strain KH17 gave ~60% of the CFE specific activity with MCA and ~30% of the CFE activity with 2MCPA, which may imply different dehalogenases associated with pellet material (membrane). Significantly higher dehalogenases specific activities were obtained with the pellet from *Methylobacterium* sp. strain KH4, which were ~5-fold higher than in the corresponding CFE (Table 4.8).

Native polyacrylamide gel electrophoresis (PAGE) of CFEs from strains KH3, KH17, KH4, stained with MCA or 2MCPA, allowed the visualization of dehalogenases. Same results obtained with both substrates in strain KH17 and KH3, producing a single dehalogenase, but two dehalogenases were present in the CFE from strain KH4 (Figure 4.10, Table 4.7).

Table 4.8 Relative dehalogenase activity in crude cell pellets of the isolated strains

Bacterial strain	Dehalogenase specific activity of the pellets from the CFEs (U/ mg protein)[a, b]	
	MCA	2MCPA
Afipia sp. strain KH3	ND	0.06
Rhizobium sp. KH31	0.04 (100)	0.01 (25)
Herbaspirillum sp. strain KH17	0.17 (100)	0.11 (65)
Methylobacterium sp. strain KH4	0.98 (100)	ND

a. A unit of dehalogenase activity is defined as the amount of enzyme required to convert 1 μmol substrate/min
b. Values in parentheses represent percentage activity relative to MCA
ND, no activity detected

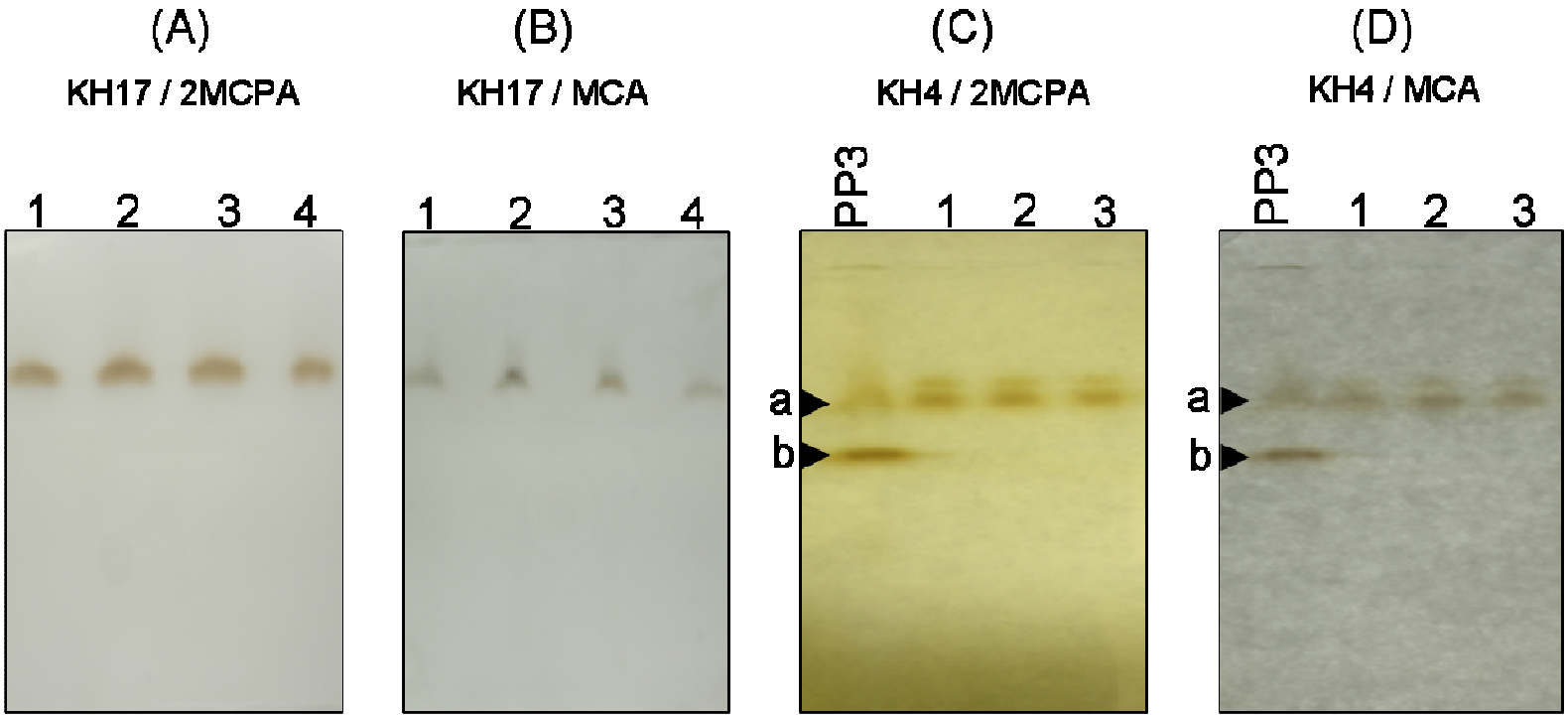

Figure 4.10 Native PAGE of cell-free extracts (CFE) stained as described in section 2.13 following incubation with 2MCPA or MCA. The isolates were grown in SBS medium containing 2MCPA as the carbon and energy source. (A) Strain KH17 stained with 2MCPA. (B) Strain KH17 stained with MCA. (C) Strain KH4 stained with 2MCPA. (D) Strain KH4 stained with MCA. Lanes (1-4) amount of CFE (100 µl per well) as follows: lane 1, 250 µl; lane 2, 200 µl; lane 3, 150 µl; lane 4, 100 µl. The lane labelled PP3 contained CFE from *Pseudomonas putida* strain PP3 produced group I and group II dehalogenases indicated by: (a) $DehI_{PP3}$ and (b) $DehII_{PP3}$.

4.4 Discussion

4.1 Analysis of sub-cultures from AS-RBTs and soil RBTs

The DGGE profiles of all AS-RBT sub-cultures showed that most of the bacterial phylotypes dominating the RBT were not successfully sub-cultured (Figure 4.4A). One exception was the *Caulobacter* sp. phylotype (S11-1/S16-1; Figures 4.1 and 4.2) originally identified in the AS-RBT (AS6 in Figure 3.2; S11-1 and S11-11 in figure 4.1). However, ultimately, this organism was not one of the purified isolates.

Failure to isolate *Caulobacter*, may be explained by its specific biochemical and/or physiological requirements which perhaps are provided by other bacteria in the original AS-RBT consortium and may have been lost during sub-culturing (Slater and Lovatt, 1984; Slater et al., 1995). Another reason may be that the colonies of this bacterium were not picked, since colonies selection of 2MCPA-degraders was random.

The absence of the phylotypes from the AS-RBT that became dominant and detectable in the sub-cultures (Figures 4.4A), may be explained by the preferential cultivation of bacteria present as minor populations in the RBT that were not detected by DGGE (Jackson et al., 1998).

The AS-RBT enrichment was dominated by *Alpha-* and *Betaproteobacteria* (Chapter 3), and the dominant phylotypes identified in DGGE bacterial community profile of the sub-cultures derived from the AS-RBT were *Alphaproteobacteria*, with relatively few *Betaproteobacteria*; *Gammaproteobacteria*; *Actinobacteria* and *Bacteroidetes* (Tables 4.1, 4.2 and 4.3). Many of these phylotypes were assigned to species previously reported in the literature to include αHA degraders from activated sludge and soil; for example, strains of *Caulobacter* sp. (Marchesi and Weightman, 2003b), *Rhizobium* sp. (Cairns et al., 1996), *Afipia* sp. (Zhang et al., 2009b), *Bradyrhizobium* sp. (Marchesi and Weightman, 2003a), *Xanthobacter* sp. (Janssen et al., 1985; Hill and Weightman, 2003). Schwarze et al. (1997) reported the isolation of a *Comamonas* species able to degrade 22DCPA, which was also able to grow on MCA, DCA, 2MCPA. A strain of *Comamonas* sp. was reported to be associated with TCA degradation (McRae et al., 2004). Strains of *Afipia* sp. isolated from drinking water have been reported to contain group I and group II dehalogenase genes, and are able

to degrade haloacetic acids (MCA, DCA, TCA) (Leach et al., 2009; Zhang et al., 2009b). Several phylotypes from AS-RBT sub-cultures were not previously reported to degrade αHA, but were known to be catabolically versatile and degrade other related compounds. For example, strains of *Arthrobacter* sp., can degrade 4-clorobenzoate (Zhou et al., 2008) and benzene (Xie et al., 2010) and have been identified in soil contaminated with heavy metals (Ellis et al., 2003) and petroleum (Juck et al., 2000). *Polaromonas* species have been associated with degradation of naphthalene and benzene (Xie et al., 2010) and has been isolated on 1,2-dichloroethene (Mattes et al., 2008). Members of the genus *Acinetobacter* were reported to degrade several pollutants, including, short-chain chlorinated aliphatic compounds (Olaniran et al., 2005), phenol (Adav et al., 2007), paraffin (Koma et al., 2001) and nicotine (Wang et al., 2011).

The observation that *Alphaproteobacteria* were the dominant phylotypes in the AS-RBT and the sub-cultures, suggests that αHA dehalogenase genes occur more frequently in this phylum, and supports the findings of Marchesi and Weightman (2003b) suggesting that αHA dehalogenases may have originated in species of *Alphaproteobacteria* (Hill and Weightman, 2003; Liu et al., 2011). Liu et al. (2011) reported that *Alphaproteobacteria* were major 2-methyl -4-chlorophenoxy acetic acid (MCPA) degraders in soil.

Gram-positive 16S rRNA phylotypes were identified in some sub-cultures (e.g. S16-15, Figure 4.2; Table 4.2), suggesting the presence of αHAs-degraders among the Gram-positive bacteria (Kerr and Marchesi, 2006; Chiba et al., 2009). There are reports of Gram-positive bacteria degrading αHAs (Tsang et al., 1988; Marchesi and Weightman, 2003a; Kerr and Marchesi, 2006); however, none of these strains have been characterized in detail. Olaniran et al. (2004) described the isolation of Gram-positive bacteria able to degrade 1,2-dichloroethane, and MCA. Kerr and Marchesi (2006) reported the isolation of Gram-positive bacteria from activated sludge able to degrade 22DCPA. Gram-positive bacteria have also been reported degrading other halogenated compounds, such as 3-chloropropionate (Scholtz et al., 1987a), and 4-chlorobenzoate (Zhou et al., 2008). Rhodococci have been isolated from various habitat such as soil and sea water, they are resistance to many toxic compounds and able to degrade many of xenobiotic compounds such as, aromatic compounds, mono

and poly aromatic hydrocarbons, phenol, aromatic acids halogenated compounds, nitroaromatics and have the ability to uptake and metabolize hydrophobic compounds (Martínková et al., 2009). Rhodococci harbour large linear and circular plasmids, which may accommodate a large number of catabolic genes (Larkin et al., 2005). The high frequency of recombination increase the adaptability and flexibility of their genome (Larkin et al., 2005), and ability to acquire new genes by horizontal gene transfer and consequently new enzymatic activities (Larkin et al., 2005; Martínková et al., 2009).

Using 16S PCR-DGGE, it was possible to follow changes in bacterial communities during their acclimation to degrade αHA (2MCPA), and also investigate effects of different concentrations of substrate on bacterial community profiles through sub-culturing. However, problems such as co-migration of DGGE fragments representing different phylotypes were evident in this study, and needed to be taken into account. Jackson et al. (1998) reported DGGE bands co-migration, even when band sequences differed by 5%, because they had same GC content (50%) and melting temperature (83^{o}C). In addition, multiple bands for a single species due to existence of multiple copies of 16S rRNA gene in one organism have been reported (Nubel et al., 1997; Wintzingerode et al., 1997; Klappenbach et al., 2000). 16S rRNA gene PCR of *Herbaspirillum* sp. strain KH17 (*Betaproteobacteria*) gave two bands on DGGE gel (Figure 4.5), which may account as separate bands of different species. Therefore, it is important to verify the identity of DGGE bands, as far as possible by sequencing, to obtain a reliable information and identification (Ferris et al., 1996).

The liquid sub-cultures derived from the AS-RBT contained a lower diversity of bacteria than the original RBT, but were relatively stable through up to three sub-cultures. However, it was only possible to purify and isolate two of the organisms on solid medium: *Afipia* sp. KH3, and *Rhizobium* sp. KH31 (*Alphaproteobacteria*).

The fact that neither of these strains dominated the original AS-RBT was consistent with previous studies where, cultivation-dependent and independent approaches have produce different results with respect to dominant bacterial species involved in pollutant biodegradation (Marchesi and Weightman, 2003b, a; McRae et al., 2004). Dunbar et al. (1997) reported that batch-culture enrichment methods are highly selective, resulting in isolation of a few microbial species from natural microbial

communities, and that cultivated bacteria obtained did not represent the natural population from which they were obtained. Watanabe et al. (1998) reported the isolation of non-dominant phenol-degrading bacteria from a consortium able to degrade this pollutant. Several reasons have been put forward to explain this, such as, different biases associated with DNA-based and cultivation-based methods for assessment of microbial communities. DNA extraction and PCR biases in assessment of microbial diversity are well documented (Wintzingerode et al., 1997; Polz and Cavanaugh, 1998). With respect to cultivation biases, the degradation of 2MCPA may require the combined activity of the consortia; for example, 2MCPA degrading bacteria may require specific nutrients provided by other members of the consortium (cross-feeding) (Slater and Lovatt, 1984; Van den Wijngaard et al., 1993). Depletion of nutrients by faster-growing bacteria can out-compete slow-growing bacteria making them difficult to detect (Davis et al., 2005). There are reports that members of *Alphaproteobacteria* are generally slow growing (Jordan, 1982; Mitsui et al., 1997; Jackson et al., 1998). This may be the reason why the pure cultures were not enriched in the AS-RBT, and that the bacteria dominating the AS-RBT (AS-6, AS-7 and AS-10, Figure 3.2; Table 3.1) were probably the faster growing primary 2MCPA utilizers, which were dependent to some extent on other organisms (possibly secondary non-2MCPA utilizers) for the ability to degrade 2MCPA. The selection of bacteria from the AS-RBT on 2MCPA agar, and further sub-culturing in 2MCPA liquid culture would be based in favour of bacteria which could degrade 2MCPA in isolation and were not inhibited by agar or other media constituents.

For the bioaugmentation of polluted sites, it is essential that the inoculated bacteria adapt to the environment and degrade the pollutant effectively under the prevailing environmental conditions. The results in this study illustrate that such bacteria were missed throughout the liquid sub-culturing process. In addition, the results showed that cultivation on agar without sub-culturing is more suitable for isolating the 2MCPA degraders.

Although the soil-RBT sub-cultures were not monitored as closely as AS-RBT, they were successful in terms of isolating two bacterial strains representing major phylotypes identified in the original enrichments; *Herbaspirillum* sp. strain KH17 (*Betaproteobacteria*), which matched a phylotype in the HS-RBT, and

Methylobacterium sp. KH4, which matched a less dominant phylotype from the LS-RBT (Figure 4.5).

DGGE profiles indicated many shared bands, indicating the same phylotypes, comparing 2 mM and 10 mM 2MCPA sub-cultures. However, some significant differences were observed in DGGE banding patterns between sub-cultures at two different concentrations of 2MCPA. Some phylotypes were able to grow only in sub-cultures containing 10 mM 2MCPA; for example, phylotypes represented by bands S11-5, S11-7, S11-8 (Figure 4.1), and bands S16-12, S16-15 (Figure 4.2). By contrast, other phylotypes, such as bands S16-4, S20-5 (Figures 4.2 and 4.3), were more dominant at 2 mM 2MCPA. This may be explained by that the growth kinetic parameters, Ks/μmax for the different bacterial species on αHAs, which may in turn reflect the dehalogenases of these organisms (Futamata et al., 2003; Viggor et al., 2008). For example, Viggor et al. (2008) studied two Pseudomonads, one of which was able to tolerate phenol as a growth substrate at much higher concentrations than the other. This was explained by the strains' differences in Ks and μmax, which reflected production of different phenol hydrolases. Futamata et al. (2003) reported that low concentrations of phenol increased the population of low Ks TCE/phenol degrading bacteria belonging to *Betaproteobacteria*. Alternatively, growth of some phylotypes could be inhibited by high concentration of 2MCPA, which could be toxic (Weightman et al., 1985).

4.2 Characterization of 2MCPA-utilizing bacteria isolated from AS- and soil-RBTs

Four new bacteria able to grow on 2MCPA were isolated and partially characterized. Strain KH3 was identified as *Afipia* sp. based on nucleotide sequence of the 16S rRNA genes (Figure 4.6). Strain KH3 was most closely related to *Bradyrhizobium* and *Oligotropha carboxidovorans* and *Afipia* sp. on the basis of 16S rRNA gene analysis. Previous studies have shown that *Bradyrhizobium* and *Oligotropha carboxidovorans* and *Afipia* are closely related and difficult to resolve on the basis of 16S rRNA gene sequences alone (Willems and Collins, 1992; Willems et al., 2001; Islam et al., 2008; Paul et al., 2010). A study to determine the taxonomic position of *Bradyrhizobium* sp. EK05 isolated in Japan, showed that analysis of 16S rRNA gene related the sequence

to the genus *Bradyrhizobium* and *Oligotropha carboxidovorans*, but sequence analysis with *gyrB* showed that the isolate related to *Bradyrhizobium* but with distinct evolutionary lineage (Islam et al., 2008). The use of information from the comparison and combination of multiple genes can give a reliable overview of closely related bacteria (Martens et al., 2008). Therefore, other genes such as *glnII* (encoding a glutamine synthesis), *recA* (encoding recombinase A protein), *gyrB* (encoding DNA gyrase subunit B), and *atpD* (encoding the B subunit of ATP synthase) (Islam et al., 2008; Noisangiam et al., 2010) have been used. Strain KH31 was identified as member of family *Rhizobiaceae* and assigned to the genus *Rhizobium* (Figure 4.6A), and strain KH17 grouped most closely with *Herbaspirillum* sp. (*Betaproteobacteria*), showing 16S rRNA 99% sequence similarity with *Herbaspirillum* strain DA1 (Marchesi and Weightman, 2003a). Strain KH4 was identified as a *Methylobacterium* sp. (Figure 4.6B).

All bacterial isolates except strain KH4 were able to grow on MCA, DCA, and 22DCPA (in addition to 2MCPA) but not TCA or 2MCBA. They all showed dehalogenase activity against a range of αHA substrates (Table 4.7), and each strain produced at least one αHA dehalogenase (Figure 4.10).

The bacterial genera isolated from this study have all been previously shown to possess the ability to degrade a wide variety of organic compounds including many halogenated xenobitics. Strains of *Afipia* sp. isolated from drinking water have been reported to contain *dehI* and *dehII* genes described by Hill et al. (1999), and to be able to degrade haloacetic acids (MCA, DCA, TCA) (Zhang et al., 2009b). *Afipia* sp. also have been also isolated from soil able to degrade 3,4-DCA (3,4-dichloroaniline) (Breugelmans et al., 2007). Strains related to *Afipia felis* have been isolated from Antarctic soil and were the first described bacteria being able to use both methanesulfonate and dimethylsulfone as sole carbon source (Moosvi et al., 2005). *Bradyrhizobium* spp. are capable of degrading a number of halogenated aliphatic and halogenated aromatic including, 2,4-dichlorophenoxyacetatate (2,4-D), and 3-chlorobenzoate (Kamagata et al., 1997; Vela et al., 2002; Gentry et al., 2004). Marchesi and Weightman (2003a) reported isolation of a strain of *Bradyrhizobium* on 22DCPA (Dalapon) from pristine soil, which contained both *dehI* and *dehII* genes (Hill et al., 1999).

Strains of *Rhizobium* sp. isolated from different environments (activated sludge and soil) reported to be able to degrade pollutants including halogenated aliphatic and aromatic compounds, including mono- and dichloropropanol, chlorophenols, chlorobenzene, and bromopropanol (Effendi et al., 2000; Bastos et al., 2002; Vela et al., 2002; Zhang et al., 2008). A strain of *Rhizobium* sp. has been isolated from soil utilizing 22DCPA, dehalogenase from this strain was able to act on MCA, DCA, 2MCPA, and 22DCPA (Berry et al., 1979). Dehalogenases from strains of *Rhizobium* have been characterized that were D-specific dehalogenases acting only on D-2MCPA (Cairns et al., 1996; Higgins et al., 2005).

Several studies have reported the isolation of members of the genus *Herbaspirillum* from various environments including soil, water, sediments. A strain of *Herbaspirillum* sp. has been isolated from stream sediment able to degrade 4-chlorophenol (Im et al., 2004). Strains of *Herbaspirillum* sp. have been reported previously growing on other chlorinated aliphatic hydrocarbon (trichloroethene) (Connon et al., 2005). Strains of *Herbaspirillum* have been previously reported to degrade αHAs. Marchesi and Weightman (2003a) reported the isolation of *Herbaspirillum* strain DA1 able to degrade 22DCPA. Cloning and sequencing of PCR amplified *dehI* gene from strain KH17 showed to be identical at the nucleotide sequence level to *dehI* of *Herbaspirillum* sp. DA1 (Figure 4.9) (Marchesi and Weightman, 2003a).

A strain of *Methylobacterium* sp. has been reported previously to utilize chloromethane and dichloromethane as the sole carbon and energy sources and possess dehalogenases (Scholtz et al., 1988b; La Roche and Leisinger, 1991; Fulthorpe et al., 1993; Janssen et al., 2002; McDonald et al., 2002). Sette et al. (2007) have reported the isolation of *Methylobacterium* sp. able to degrade oil. Strains of *Methylobacterium* sp. have been previously reported to degrade αHAs such as DCA, 2MCPA and 22DCPA (Omi et al., 2007; Jing and Huyop, 2008; Zhang et al., 2009b). *Methylobacterium* sp. HJ1 isolated on 22DCPA was not able to utilize MCA, DCA, but the dehalogenase from this strain was able to act on MCA, DCA, 2MCPA and 22DCPA (Jing et al., 2008). A strain of *Methylobacterium* sp. strain CP13 isolated from bleached Kraft pulp and paper-mill effluent grew well and completely dechlorinates both MCA and DCA (Fulthorpe and Allen, 1995).

Methylobacterium sp. strain KH4 degraded 2MCPA completely; however, neither *dehI* nor *dehII* were detected, although dehalogenase PAGE showed that strain KH4 contain at least two dehalogenase bands (Figures 4.8 and 4.10). Hill et al. (1999) reported that a number of dehalogenases were not amplified using their degenerate *deh* primers. Several dehalogenases, *dehH1* from *Delftia acidovorans* strain B (Kawasaki et al., 1992), *dehL* from a *Rhizobium* sp. (Cairns et al., 1996) and *dehII* from *Rhizobium* NHG3 (Higgins et al., 2005), and several groups of bacteria isolated on 22DCPA (Kerr and Marchesi, 2006) would not be detected using these primers. Other workers reported that *Methylobacterium* contained *deh* which was not amplifiable using group I (*dehI*) and group II (*dehII*) specific primers described by Hill et al. (1999). *Methylobacterium* strain PAWDI isolated on DCA and able to grow on MCA, only contained a *dehI* gene which was not amplified using *dehI* and *dehII* primers (Zhang et al., 2009b), but amplified by an alternative PCR primer sets previously described to amplify *dehI* (DL-DEXmb) from a *Methylobacterium* sp. CPA1 (Omi et al., 2007). Therefore, these results suggest that the dehalogenases of *Methylobactereium* sp. KH4 may not relate to either group I or group II dehalogenase families, and may be novel.

Strain *Rhizobium* KH31 may contain a stereospecific dehalogenase which acts only on one isomers of the 2MCPA either L- or D-2MCPA, since only 50% dechlorination of the 2MCPA was observed. However, PCR products with both *dehI* and *dehII* genes were obtained from strain KH31 (Figure 4.8).

Dehalogenase activity was also observed in pellet fractions of all the isolates (Table 4.8), suggesting evidence of membrane-associated αHA dehalogenases. To date no membrane-associated αHA dehalogenases have been described in the literature, but *Rhodococcus erythropolis* Y2 was shown to carry a second haloalkane dehalogenase of the oxygenase type which was found to be membrane-associated enzyme (Armfield et al., 1995). Also *Rhizobium* NHG3 was reported to produce membrane-associated haloalcohol dehalogenase activity (Effendi et al., 2000), and this strain was also able to dechlorinate 2MCPA showing two 2MCPA dehalogenases on gel. A reductive dehalogenases from *Desulfomonile tiedjei* DCB-1 and other halo-respiring bacteria are present in the membrane (Ni et al., 1995). Similarly, trichloroethene (TCE) dehalorespiration by anaerobic *Dehalococcoides ethenogenes* was also found to be

membrane-associated dehalogenases (Magnuson et al., 2000). A membrane-associated monooxygenase was found in *Mycobacterium* sp., able to degrade chlorinated phenol (Uotila et al., 1992).

Differences between the isolates' dehalogenase substrate specificities (Table 4.7) and their respective αHA growth substrate specificities (Table 4.6) may be explained by a number of factors. For example, the observation that strain KH4 only grew on 2MCPA, but showed dehalogenase activity with at least three other αHA substrates (Table 4.7) could be: (a) strain KH4 is not being able to utilize the dehalogenated products of MCA (glycolate), DCA (glyoxylate) or 22DCPA (pyruvate); (b) toxicity of the non-growth substrates; (c) lack of a transporter protein (permease) for the non-growth substrates; and/or (d) inability of the non-growth substrates to induce expression of the dehalogenase (s) in strain KH4. Characterization of *Pseudomonas putida* strain PP3 has demonstrated that inability to utilize some dehalogenation products and substrate toxicity explains, to some extent, discrepancies between dehalogenase substrate specificity and growth of this strain with different αHAs (Weightman and Slater, 1980; Weightman et al., 1985).

Although, either *dehI* and/or *dehII* gene sequences were amplified from all isolates except strain KH4, further work would be needed to confirm that these genes encoded for the dehalogenases produced by these strains, since it is possible that some of the PCR products (Figure 4.8) might be derived from silent *deh* genes. Previous reports indicated the presence of cryptic or silent dehalogenase genes in some bacteria that can be switched on; for example, a cryptic dehalogenase gene in strain *P. putida* PP3 (Hill et al., 1999) and a cryptic L-isomer specific dehalogenase in *Agrobacterium* sp. RS5 (Köhler et al., 1998). Tsang and Sam (1999) also reported the presence of a cryptic dehalogenase in *Burkholderia cepacia* MBA4 active towards L- isomer 2MCPA.

5. General Discussion

The biodegradation of chlorinated pollutants and the ability of bacteria to grow on these xenobiotic compounds, using them as carbon and energy sources, have been intensively studied (Jensen, 1957; Goldman, 1965; Janssen et al., 1985; Weightman et al., 1985; Aelion et al., 1987; Marchesi and Weightman, 2003a). However, most of our knowledge about xenobiotic biodegradation has been obtained from isolation and characterization of bacteria which have been isolated using conventional cultivation methods (Jensen, 1957; Weightman and Slater, 1980; Klages et al., 1983; Janssen et al., 1985; Marchesi and Weightman, 2003a), as a result of which enzymes such as dehalogenases, and metabolic pathways responsible of degradation have been characterized (Motosugi et al., 1982c; Liu et al., 1994; Nardi-Dei et al., 1999). More recently, cultivation-independent methods, based on analysis of PCR amplified 16S rRNA genes, have been used widely and showed changes in the community and dominance of certain members following xenobiotic degradation (Jackson et al., 1998; Muyzer and Smalla, 1998; Marchesi and Weightman, 2003b; McRae et al., 2004; Morimoto et al., 2008). However, few studies have involved combinations of cultivation-dependent and independent approaches to investigate diversity of bacterial populations during degradation of xenobiotics. Therefore, this study used such a combination of approaches to investigate the diversity and distribution of αHA (2-chloropropionic acid, 2MCPA) degraders in environmental samples.

The enrichment cultures based on the OECD's Ready Biodegradation Tests (RBTs) showed 2MCPA degradation almost immediately after inoculation, and 2MCPA was readily degradable in both AS (activated sludge)-RBT and soil RBTs by the indigenous microbial communities in these inocula. Results from the soil RBTs showed that concentration of inoculum had a significant effect on the 2MCPA biodegradation with respect to both the mineralization of the substrate, and the bacterial community structure. Lower concentration of the inoculum (LS-RBT) showed a significant longer lag phase and complete degradation of the substrate was six fold longer than for the HS-RBT.

Community acclimation to degrade a persistent compound is generally considered to involve the following (Becker et al., 2006; Gaillard et al., 2008): (a) induction of catabolic genes; (b) mutation, rearrangement and genetic exchange; (c) cometabolism

e.g. addition of degradable carbon source; (d) enrichment of organisms from the inoculum to a level where biodegradation is detectable. However, since degradation of 2MCPA was completed between 10, 20 and 60 days in the RBTs studied here, it is possible that all of these factors could be involved in acclimation of the bacterial community to degrade this compound.

The time taken to enrich of dehalogenating organisms was related to the inoculum concentration used in the RBT, lower inoculum concentration influenced the level of specific degraders within the starting community as seen by the presence or absence of bacterial population and *dehI* and *dehII* PCR products, thus increasing the lag phase of the enrichment.

Biodegradation requires the enrichment of a small population of degrading organisms to a level where degradation is detectable (Spain and Van Veld, 1983; Ramadan et al., 1990; Ingerslev et al., 2000). Several studies with environmental samples and isolated microorganisms have described reduced degradation of organic contaminants at low inoculum concentration (Spain et al., 1980; Ramadan et al., 1990). The time required for induction of catabolic genes and their translation into protein is commonly cited as an explanation for the lag phase during microbial acclimation (Becker et al., 2006). Gene expression may, in some cases, be linked to delayed adaptation of inoculum. However, since the lag phase observed prior to substrate dechlorination measured in days, there for, induction of catabolic genes is unlikely to be a factor of the long lag phase, since enzyme induction is usually take only minutes or hours (Richmond, 1968).

Another explanation is that production of dehalogenases required for 2MCPA degradation may be suppressed by the presence of other substrates in the inoculum, which may delay adaptation of the inoculum to the chlorinated substrate (Field and Sierra-Alvarez, 2008). Carbon catabolite repression (CCR) is regulatory phenomenon where the presence of a preferred carbon source prevents the expression and activity of catabolic system that enable the use of secondary substrates (Görke and Stülke, 2008). Becker et al. (2006) reported that rapid growth of 3-chlorobenzoate only occurred when readily biodegradable endogenous substrates was depleted, which resulted in delay of 3-chlorobenzoate degradation.

The long lag phase observed prior to 2MCPA degradation in the LS-RBT (Chapter 3) may also have partly resulted from the toxicity of the substrate which may have delayed the community acclimation in the LS-RBT. Some of αHAs, such as MCA and DCA, are known to be toxic to bacteria (Weightman et al., 1985); however, the substrate concentration of the αHA used in this study was at a concentration lower than those previously been reported to be toxic.

Under the low inoculum size condition, 2MCPA degradation was accomplished in two phases of dechlorination and this biphasic response was attributed to the enrichment of *dehII* genes followed by *dehI* genes, associated with degradation of L-2MCPA and D-2MCPA, respectively. This was probably a result of abundance and diversity of the *dehI* and *dehII* gene families in the inoculum, and that the lower inoculum size resulted in decreasing *dehI* genes, which have been reported to be less diverse and abundance than *dehII* genes (Hill et al., 1999). Therefore, the biphasic dechlorination curve observed in the LS-RBT with the low inoculum size could be explained in terms of bacterial species succession in the 2MCPA degrading community, and associated dehalogenase stereospecificity.

The results in this study confirmed and extended previous studies (Hill and Weightman, 2003; Marchesi and Weightman, 2003b) that showed the diversity of bacteria and dehalogenases in the RBT communities was greater than normally seen in conventional, cultivation-based studies. Even though the focus on the uncultivable fraction by the methods used (16S rRNA gene PCR-DGGE) to detect the bacterial population, neglects the organism viability (Keer and Birch, 2003), cultivation and isolation of αHA-degraders from environment after complete degradation of the substrate (Tsang et al., 1988; Hasan, 1994), misses intermediate stages during community acclimation. For example, in the LS-RBT end-point sampling would have missed the succession of *Comamonas* phylotype (enriched in phase 1) by the *Methylibium* phylotype (appeared in phase 2).

This study used a DNA-based approach; however, the identification of catabolic genes transcription from RNA analysis is important, since DNA analysis alone would detect both active and inactive microbial populations, as well as exogenous DNA free released from microbial cells, which is relativly stable in the environment (Keer and Birch, 2003; Bodrossy et al., 2006; Nielsen et al., 2007). Unfortunately, this study was

initiated before the development of a technique uses propidium monoazide (PMA) to distinguish between DNA from live cell and DNA from the surrounding environment (Nocker et al., 2007). Nevertheless, changes in the 16S rRNA and *deh* genes profiles were readily detected in the RBTs and the sub-cultures, suggesting a dynamic bacterial community with succession of species, and indicating competition between organisms within the community. The results from DNA analyses also suggested that the bacterial community enriched were active and have a role in the degradation process (i.e. fluctuation in DNA profiles observed in this study would not be expected if the community was essentially inactive or dominated by dead cells). A study on linuron herbicide (N-(3,4-dichlorophenyle)-N-methyle urea) mineralization, showed that the results of bacterial communities studied by PCR and reverse transcription-PCR DGGE 16S rRNA were similar (Sørensen et al., 2005), and a recent study suggested that DNA and RNA (cDNA) profiles (e.g. 16S rRNA DGGE) were almost identical (Leggett, 2010). However, it remains desirable to analyse RNA to monitor bacterial activity (Revetta et al., 2010) in particular, dehalogenase genes which are not necessarily co-linear with their DNA template (Hill et al., 1999). Therefore, any future work to investigate capacity of bacteria to degrade αHAs should also include RNA analyses focusing on dehalogenase genes mRNA and 16S rRNA.

Genetic exchange involving lateral or horizontal gene transfer (HGT) of mobile genetic elements (MGE) is known to play a key role in the acclimation of bacterial population to degrade xenobiotics, due to acquisition of catabolic genes. MGEs include transferable plasmids, insertion sequences, transposons, integrons and genomic islands (Gaillard et al., 2008; Juhas et al., 2009; Miyazaki and Van Der Meer, 2011a). Dejonghe et al. (2000) reported that plasmid transfer was involved in the adaptation of a soil community to degrade the chlorinated herbicide 2,4-D. Plasmids are well known to contribute to the wide dissemination of catabolic transposons (Top et al., 2002) which promotes the evolving of novel catabolic pathways by genetic rearrangement (Van der Meer et al., 1992), and HGT is associated with the formation of genomic islands (GEIs), which often carry insertion elements and transposons. GEIs have been shown to be capable of integration into the chromosome of the host, as well as excision and transfer to new hosts by transformation conjugation and transduction involving in dissemination of variable genes, including antibiotic resistance, pathogencity, symbiosis, and catabolic genes

(Beaber et al., 2002; Toussaint et al., 2003; Mohd-Zain et al., 2004; Gaillard et al., 2008). Antibiotic resistance represents one of the most frequent and well studied traits associated with GEIs (Juhas et al., 2009). A number of GEIs referred to as ICE or integrative conjugative elements have been identified in *Beta* and *Gammaproteobacteria* which carry gene clusters for the degradation of chlorinated and nitroaromatics or biphenyl (Toussaint et al., 2003; Gaillard et al., 2008). Recently, Miyazaki and Van Der Meer (2011a) reported that the ICE*clc* of *Pseudomonas knackmussii* B13, involved in degradation of 3-chlorobenzoate, carried two separate origins of transfer (oriT1 and oriT2), and that the dual origin of transfer system may provide an evolutionary advantage for distribution, which can be transferred to recipient cell via conjugation and integrates into the recipient chromosome at a specific sites (Miyazaki and van der Meer, 2011b). ICE*clc* represents a large group of elements that readily transfer between different species of *Proteobacteria* and confer to its host the capacity to metabolize new compounds, and are major contributors to bacterial evolution (Miyazaki and Van Der Meer, 2011a). For example, transfer of the *clc* elements in *Pseudomonas aeruginosa* strain PAO1 enabled the strain to grow on 3-chlorobenzoate and 2-aminophenol (Gaillard et al., 2008).

Brokamp et al (1997) found several dehalogenase genes to be present on mobile genetic elements. For example, plasmid pUO1 from *Delftia acidovorans* strain B carries two haloacids dehalogenases genes (*dehH1* and *dehH2*) on the two transposons, Tn*Had1* and Tn*Had2* (Sota et al., 2002). Hill and Weightman (2003) also reported an association of HGT between plasmids and bacterial adaptation to degrade αHA compounds, in *Ralstonia* strains which also carried IS elements identical to IS*Ppu12* found in *Pseudomonas putida* PP3 (Hill and Weightman, 2003).

Bacteria may also adapt to degrade a substrate by activating silent genes already present in their genome (Mattes et al., 2010). Gene activation may be a result of insertion sequence activity, e.g. promoterless phenol degradation genes, *pheBA*, can be activated by the adjacent insertion of IS*1411*, which contain a promoter (Kallastu et al., 1998; Peters et al., 2004). Activation of silent dehalogenase genes in response to starvation has been reported previously (Hill et al., 1999), but little information is known about when, where, between which species, or at what frequencies such mechanism occur (Top and Springael, 2003).

In the present study comparison between the data obtained using cultivation-independent and cultivation based methods indicated that differential selection of community members (different phylotypes) occurred during biodegradation of 2MCPA. Two bacterial strains were successfully isolated from the soil-RBTs which represented phylotypes in the enriched communities, but the bacteria enriched from AS-RBT sub-cultures could not be identified in the original RBT. Several factors may explain these discrepancies between results from cultivation-independent and cultivation-based approaches. For example, the bacteria obtained on plates and not detected by molecular method might not have been major components of the environmental sample analysed (Watanabe et al., 1998). Alternatively, they may have been missed in DNA analyses due to PCR biases when amplifying community DNA (preferential amplification), or different lysis efficiency may have occurred during extraction of DNA from the samples prior to PCR amplification of 16S rRNA gene (Wintzingerode et al., 1997). Also, it has been reported that agar has toxic effect on some groups of bacteria, and that using other solidifying reagent can improve cultivability (Davis et al., 2005; Tamaki et al., 2005). Tamaki et al. (2005) reported that use of gellan gum instead of agar increased cultivability of *Betaproteobacteria*. The enriched bacteria in the RBT might be dependent for growth on interactions with other bacteria (members of the consortia), as found in an earliar study where 2MCPA was degraded by a group of metabolically cooperating bacteria in a continous-flow (chemostat) culture (Slater et al., 1997).

It was notable that the isolates which grew best on 2MCPA were all *Alpha* or *Betaproteobacteria*, and they were similar in many respects to bacteria previously isolated on αHAs (Marchesi and Weightman, 2003a; Higgins et al., 2005; Omi et al., 2007). However, it seems unlikely that bacteria in these orders are more easily cultivable on the SBS-based media used in this study than the other *Proteobacteria* that dominated the AS-RBT. For example, *Gammaproteobacteria* are readily cultivated on this medium (Slater et al., 1979; Weightman and Slater, 1980; Hill and Weightman, 2003; Marchesi and Weightman, 2003b). Despite this, none of the isolated strains was related to *Gammaproteobacteria*, a result which is surprising and inconsistent with the previous reported isolations of *Gammaproteobacteria* on αHAs (Weightman et al., 1979; Schneider et al., 1991; Barth et al., 1992; Nardi-Dei et al., 1994; Hill and Weightman, 2003).

The difference in outcome of the two approaches found in this study does not mean that the isolation of bacteria is not an apprropriate method for investigating αHA degradation. Rather, the purpose was to compare the two approaches and to show that relying on results using one approach should be avoided, since techniques such as liquid sub-culture often fails to isolate the actual degraders in the environment (Morimoto et al., 2008). Results of this study indicates the potential usefulness of molecular techniques application when studying xenobitics degradation, giving more information on the diversity of bacteria able to degrade the compounds from that obtained using cultivation methods. Moreover, the result suggest that combination of molecular method based on PCR-DGGE, and cultivation methods greatly helps in investigating relationships between enriched bacteria in RBT system and their representatives in pure cultures. Thus, the combination of molecular-based and cultivation-based methods complement each other, using molecular techniques to find novel bacteria able to degrade xenobiotics, and then finding or understanding the nutrient requirements, growth conditions most suitable for the isolation of αHA-degrading bacteria from the environment (Palleroni, 1997). A study performed by Juck et al. (2000) showed similarly complementary results using cultivation-independent and dependent approaches when analysing soil contaminated with petroleum hydrocarbon.

This study supports others showing that enrichment cultivation on chlorinated organic compounds results in the selection of bacterial communities consisting of several taxa which act on the αHA substrate in concert, rather than a single dominant species (Slater et al., 1997). Using microbial consortium rather than pure cultures for bioremediation is more advantageous, as it provides metabolic diversity needed for field applications (Rahman et al., 2002; Mattes et al., 2010). Alisi et al. (2009) reported complete degradation of diesel oil using a consortium made of selected native strains (Alisi et al., 2009). It has been reported that a consortium metabolized atrazine faster than individual species, (through inter-species metabolic interaction within the consortium (De Souza et al., 1998). Alvey and Crowley (1996) reported that bioaugmentation with an atrazine-mieralizing consortium greatly enhanced the rate of atrazine mineralization.

Understanding the biodegradation process of αHAs and identification of bacteria which can degrade xenobiotic halogenated compounds is important in developing practical biological treatment in waste water and sediments contaminated with these compounds, and perhaps other halogenated compounds. Studies involving biodegradation under controlled condition (in the laboratory) are useful in providing information about rates of degradation of a compound, to estimate cost and duration of treatment, measure effectiveness of bioremediation under predetermined condition (Balba et al., 1998). To improve the biodegradation process, in addition to requiring competent organisms able to degrade the contaminating substrate, physical and environmental parameters, such as temperature, oxygen availability, water content, pH, toxic pollutant in the contaminated site, protozoal predation, also influence mineralization of pollutants (Vainberg et al., 2009; Tyagi et al., 2011).

Bioremediation is a promising economic, eco-friendly technology for the clean up of terrestrial and aquatic ecosystems contaminated with organic pollutants. The main current strategies for bioremediation are: natural attenuation (or self-remediation), biostimulation, and bioaugmentation, some examples of which are outlined below.

Rapid biodegradation of benzene, toluene and xylene contaminated ground water by natural attenuation was reported by Takahata et al. (2006), and bacteria that grow on 1,2-dichloroethene (DCE), isolated from contaminated and uncontaminated environments, suggest that they were influential in natural attenuation of these compounds (Mattes et al., 2010). Biostimulation, involved addition of nutrient supplements such as fertilizer to provide additional nitrogen and/or phosphorous so as to stimulate nutrient-limited native microbial populations (Delille et al., 2004; Tyagi et al., 2011). Sarkar et al. (2005) reported that biodegradation of petroleum hydrocarbon in contaminated soils was enhanced by up to 96% after addition of nutrient rich-organic matter from treated domestic sewage and inorganic fertilizer rich in nitrogen and phosphorus. The most commonly used options for bioaugmentation are: addition of pre-adapted pure bacterial strains; addition of pre-adapted consortium; introduction of genetically engineered bacteria; and addition of biodegradative relevant genes packaged in a vector to be transferred by conjugation into indigenous microorganisms (Tyagi et al., 2011). Semprini et al. (2009) showed that bioaugmentation with butane-enrichment cultures, containing two strains of

Rhodococcus, was effective in enhancing the cometabolic treatment of 1,1,1-trichloroethane (1,1,1TCA) and 1,2-dichloroethane (1,2DCA).

From a practical perspective, the results reported in this study suggest that many bacteria in soil and activated sludge that can degrade αHAs, could have substantial benefits for water and soil contaminated with these compounds. For example, *Afipia* and *Methylobacterium* have been reported to commonly occur in drinking water and may suppress the concentration of haloacids in drinking water (Leach et al., 2009; Zhang et al., 2009a).

Also, the αHA-degrading bacteria isolated in this study are known to establish close associations with plants (Valverde et al., 2003). All of the isolated strains are related to nitrogen-fixing taxa that posses the ecological feature of living in the rhizosphere soil and as endosymbionts of diverse legume plants; e.g. in root nodules (Lafay and Burdon, 1998; Sy et al., 2001; Im et al., 2004; Rivas et al., 2004; Vinuesa et al., 2005). This has possible implications for the use of biostimulation on agricultural contamination sites and suggests that the bacteria isolated in this study might be of use in bioaugmentation or rhizoremediation mediated clean up of agricultural or other contaminated sites, through plant-bacteria association.

Methylobacterium sp. strain KH4 now can be used to explore the genome of the organism, sequencing of its dehalogenase genes, and development of indicator specific primers targeting the dehalogenase genes, as well as being potentially useful as reference to investigate dehalogenating metabolism to understand the novel dehalogenase (s) in strain KH4. *Methylobacterium extorquens* DM4 is able to grow on dichloromethane through expression of a glutathione-dependent dehalogenase, DcmA (Muller et al., 2011).

The dehalogenase (s) of strain KH4 should be further investigated to compare them with other group I dehalogenases.

Whilst this study has provided insight into the acclimation of bacterial communities to degrade halogenated xenobiotics, and that it is difficult to cultivate most relevant bacteria, development in cultivation-independent techniques show promise to improve further our understanding in microbial adaptation to degrade pollutants. Metagenomic techniques, sequencing genomic DNA directly from microbial communities in the

natural or contaminated environments, provide insights into the functional, ecological and evolutionary patterns of important genes from various environments (Handelsman et al., 1998; Streit and Schmitz, 2004; Demaneche et al., 2009; Suenaga et al., 2009). More recently, next generation sequencing (NGS) have been developed to directly determine the whole collection of genes within an environmental samples and to understand community structure (genes/species distribution) and function (catabolic) by identifying and characterizing novel gene clusters (Ansorge, 2009; Demanèche et al., 2009). NGS could provide a more detailed picture of dehalogenase diversity present in environments, and could also be used to identify dehalogenase clusters to understand regulation of αHA catabolism. Stable isotope probing (SIP) of αHA substrates could be used to identify active members of the bacterial community (i.e. identification of active bacterial population in complex natural environments (Borodina et al., 2005; Osaka et al., 2008). SIP provides insights into which physiological groups of microorganisms are present, and which metabolic pathways may be operating, without the need for cultivation of isolates or prior PCR sequencing (Mattes et al., 2010).

6. Conclusions

In this study it was hypothesized that molecular genetic analysis of the bacterial community could be used to link community composition and its function. Using cultivation-independent methods, based on analysis of PCR amplified 16S rRNA genes, it was possible to follow the enrichment of different phylotypes, changes in community and dominance of certain members and corresponding dehalogenase genes (*dehI* and *dehII*) in the RBTs following exposure to 2MCPA. 16S rRNA PCR-DGGE analysis of bacterial community in the RBTs revealed enrichment of different phylotypes or species that were either not detected or not dominant in the inoculated community.

The hypothesis that higher inoculum concentration would carry greater degradative ability for 2MCPA, resulting in quicker degradation of 2MCPA, whilst lower concentration would reduce degradative ability resulting in significant slower degradation of 2MCPA. Results from soil RBTs (HS & LS) showed that the concentration of inoculum had a significant effect on the 2MCPA biodegradation and

the bacterial community composition. Lower concentration of the inoculum (LS-RBT) showed a significant longer lag phase and degradation of the substrate was quantitavily and qualitvely affected.

It was also hypothesised that bacterial community analysis using cultivation dependent methods would result in reducing the diversity of 2MCPA degraders and could result in the isolation of non representative strains from the RBTs. Bacteria capable of 2MCPA biodegradation from the RBTs were enriched and isolated. Two bacterial strains were isolated from the AS-RBT on solid medium: *Afipia* sp. KH3 and *Rhizobium* sp. KH31. However, nether of these strains dominated the original AS-RBT (i.e. cultivated bacteria obtained did not represent the community which they were obtained). By contrast, the soil RBT subcultures were successful in terms of isolating two bacterial strains, *Herbaspirillum* sp. KH17 and *Methylobacterium* sp. KH4, representing phylotypes identified in the original enrichments.

Thus the results from this study confirmed that the diversity of dehalogenases and αHA-degrading bacteria is best determined by cultivation-independent methods, and showed that cultivation on agar without sub-culturing in liqud media is more suitable for isolating 2MCPA degraders.

7. References

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