AstraZeneca

Bacterial Community Adaptation To Chlorinated Pollutant Challenge: Implications For Ready Biodegradation Testing

A thesis presented by

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Abstract

Microbial biodegradation represents a major route via which xenobiotic compounds may be removed from the environment. Bacteria, in particular, have evolved an enormous diversity of catabolic capabilities allowing them to degrade some of the most toxic and recalcitrant compounds known, many of which contain carbon-halogen bonds. The Organisation for Economic Co-operation and Development (OECD) has developed a tiered system of biodegradation tests designed to assess a chemicals environmental persistence. The ready biodegradation tests (RBTs) are the first to be conducted under this system and are designed to be a rapid, simple and cost effective method of screening chemicals, to identify potentially problematic, recalcitrant compounds that will require costly higher tier tests. However, the RBTs have previously been shown to give highly variable or falsely negative results; a characteristic commonly attributed to the use of environmental samples such as activated sludge, which have highly variable microbial composition, as an inoculum.

The α-halocarboxylic acids (αHAs) are a group of halogenated organics which are degraded by two evolutionary distinct dehalogenase families (*dehI* and *dehII*) that show different stereospecificities. Despite intensive cultivation-based research, little is known about bacterial community adaptation following an αHA challenge. Therefore, this study aimed to investigate the RBT outcome as a function of the bacterial inoculum during adaptation to degrade the αHAs dichloroacetic acid (DCA), trichloroacetic acid (TCA) and 2-monochloropropionic acid (2MCPA). A nucleic acid based cultivation-independent approach was used to study the bacterial composition (16S rRNA gene) and catabolic capability (αHA dehalogenase genes) of RBT inocula.

The αHAs investigated were ranked, in order of greatest recalcitrance; TCA>2MCPA>DCA. DCA degradation was associated with enrichment of a *Ralstonia* (*Betaproteobacteria*) like phylotype and *dehII* expression. TCA degradation in replicate RBTs was consistently associated with a *Bradyrhizobium* (*Alphaproteobacteria*) like phylotype and *dehI* gene expression, regardless of the inoculum concentration used, suggesting that TCA imposes a highly selective pressure on the community. TCA degradation was affected by the inoculum concentration, as the rate of degradation was slower at the lowest inoculum concentration used, implying that TCA degradation was accomplished by cometabolism.

2MCPA was associated with various *Alpha-*, *Beta-* and *Gammaproteobacteria*, and the presence and expression of both *dehI* and *dehII* genes. Biodegradation of 2MCPA was strongly influenced by inoculum concentration. At lower inoculum concentrations, 2MCPA was commonly associated with a biphasic dechlorination curve, which has not been reported previously. Biphasic dechlorination curves were attributed to the enrichment of an initial degrading organism, always associated with *dehII* expression, which was succeeded following 50% dechlorination by a second organism, in association with *dehI* expression, which were supposed to act sequentially on the L- and D-2MCPA isomers, respectively. The delayed enrichment of a *dehI* containing organism was attributed to their low environmental abundance, relative to *dehII* containing organisms.

Two new xenobiotic degrading organisms, *Pseudomonas* sp. strain ML1 and *Rhodococcus* sp. strain R1 (the first *Actinomycete* reported with activity against αHAs) were isolated during this study and characterised phylogenetically, and according to their dehalogenase production.

This study highlights the utility of cultivation-independent methods to link more precisely community structure and function during adaptation to degrade xenobiotic compounds.

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Abbreviations

2MCPA – 2-Monochloropropionic Acid

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

2,4-D – 2,4-Dichlorophenoxyacetic Acid

αHA – α-Halocarboxylic Acid

γ-HCH – γ-Hexachlorocyclohexane (Lindane)

BLAST – Basic Local Alignment Search Tool

BOD – Biological Oxygen Demand

cDNA – Copy DNA

CFE – Cell Free Extract

CHQ – Chlorohydroquinone

CO – Carbon Monoxide

CO_2 – Carbon Dioxide

CODH – Carbonmonoxide Dehydrogenase

CPO – Chloroperoxidase

DAPI – 4',6-Diamidino-2-Phenylindole

DCA – Dichloroacetic Acid

DDT – Dichlorodiphenyltrichloroethane

DEPC – Diethylpyrocarbonate

DGGE – Denaturing Gradient Gel Electrophoresis

dNTPs – Deoxyribonucleotide Triphosphates

DOC – Disolved Organic Carbon

DTT – Dithiothreitol

ECHA – European Chemicals Agency

EDTA – Ethylenediaminetetraacetic Acid

EU – European Union

GI – Genomic Island

HAD – Haloacid Dehalogenase

HGT – Horizontal Gene Transfer

ICE – Integrative and Conjugative Element

IPTG – Isopropyl B-D-1-Thiogalactopyranoside

IS – Insertion Sequence

MCA – Monochloroacetic Acid

MCS – Multiple Cloning Site

MGE – Mobile Genetic Element

OECD – Organization for Economic Co-Operation and Development

PAGE – Polyacrylamide Gel Electrophoresis

PCB – Polychlorinated Biphenyls

PCE – Tetrachloroethene

PCP – Pentachlorophenol

PCR – Polymerase Chain Reaction

RAPD – Random Amplified Polymorphic DNA

RBT – Ready Biodegradation Test

RDP – Ribosome Database Project

REACH – Registration, Evaluation And Authorisation Of Chemicals

RT – Reverse Transcriptase

SBS – Standard Basal Salts

SCAS – Semi-Continuous Activated Sludge System

SSCP – Single Strand Conformation Polymorphism

TAE – Tris Acetic Acid EDTA Buffer

TCA – Trichloroacetic Acid

TCDD – 2,3,7,8-Tetrachlorodibenzo-P-Dioxin

TCHQ – Tetrachlorohydroquinone

TE – Tris EDTA

TEMED – Tetramethylethylenediamine

TGGE – Temperature Gradient Gel Electrophoresis

$ThCO_2$ – Theoretical CO_2 Production

ThOD – Theoretical Oxygen Demand

tRFLP – Terminal Restriction Fragment Length Polymorphism

UPGMA - Unweighted Pair-Group Method with Arithmetic Averages

WWTP – Waste Water Treatment Plant

1. General Introduction

1.1 Xenobiotics and biodegradation

A chemical is generally considered to be xenobiotic if it is introduced into an environment in which it is entirely novel, or where its concentration far exceeds that normally present (Hutzinger and Veerkamp, 1981), where it may have deleterious effects on indigenous biota and pose a threat to human health (Ahtiainen et al., 2003; Paixão et al., 2006). The elimination of chemicals from the environment may be accomplished via several processes, including abiotic degradation, photolysis and adsorption, though the principal route for chemical elimination is microbial biodegradation (Reuschenbach et al., 2003). Bacteria and fungi are the driving forces in environmental nutrient cycling, and accounting for up to 90% of organic matter decomposition (Six et al., 2006; McGuire and Treseder, 2010). Microbes have also adapted to degrade many xenobiotic compounds, and microbial biodegradation represents a major rout via which xenobiotic compounds may be removed from the environment. Thus, the biodegradation rate of chemical pollutant will influence its environmental concentration, and the degree of exposure of a given environment to that chemical (Ahtiainen et al., 2003; Paixão et al., 2006).

1.2 Registration, Evaluation and Authorisation of Chemicals (REACH)

It is vitally important that all chemical production is subject to rigorous safety testing to ensure the safety of suppliers, customers and the environment. To this end, the European Union's (EU's) registration, evaluation and authorisation of chemicals (REACH) legislation entered into force on the 1 June 2007, refining and effectively replacing around 40 previous directives (Foth and Hayes, 2008). Its principal aim is "to ensure a high level of protection for human health, especially the health of workers, and the environment" through the better and earlier identification of the properties of chemical substances whilst enhancing the capability and competitiveness of the EU chemicals industry (Regulation, EU. (EC) 1907/2006). In practice, this requires the registration of all chemicals produced or imported into the EU at quantities greater than one tonne per year. Data regarding various properties of chemicals are held on a central database by the new European Chemicals Agency (ECHA) and used to govern their future use. A crucial element of REACH is determining the environmental persistence of chemicals by measuring their

biodegradability (Ahtiainen et al., 2003; Rudén and Hansson, 2009). The biodegradation testing scheme under the REACH legislation is outlined in Table 1.1.

Table 1.1 Biodegradation testing under the REACH scheme

Test category	Metric tons/year				
	<1	>1	≥ 10	≥ 100	≥ 1000
RBT[a]	No	(No?)[c]	Yes	Yes	Yes
Simulation[b]	No	No	No	(Yes?)[d]	(Yes?)

a. RBT = Ready Biodegradation Test. b. Simulation tests = higher tear tests designed to reflect a specific environmental compartment (see section 1.3). c. (No?): testing may be required according to certain criteria. d. (Yes?): testing may be waived according to certain criteria. Adapted from Ruden and Hansson (2009).

1.3 OECD Biodegradation Testing

In order to assess biodegradability, the OECD employs tests of varying levels of complexity, cost and environmental realism in a tiered system of three categories; tests for ready biodegradability, tests for inherent biodegradability and simulation tests (Reuschenbach et al., 2003). Due to the extremely large number of chemicals in use today, biodegradation testing requires a method which keeps cost to a minimum whilst providing sufficient information for protecting the environment.

Many chemicals entering the environment do so via aquatic systems, ultimately entering waste water treatment plants (WWTP), and consequently, most biodegradation test are conducted in aquatic phases using activated sludge as an inoculum (Forney et al., 2001; Reuschenbach et al., 2003; Paixão et al., 2006). Firstly, a chemical will be examined in a test for ready biodegradability. Ready biodegradability tests (RBTs) are stringent tests conducted under aerobic conditions with relatively high concentrations of test substance (2 to 100 mg/l), where biodegradation is monitored by non-specific parameters (see section 1.3.1 below). Negative or conflicting RBT results imply a more persistent chemical and therefore require further investigation using higher tier tests, such as the environmental simulation tests. Simulation tests are designed to be a more accurate reflection of a given environmental compartment using environmentally relevant conditions. Simulations include both aerobic and anaerobic tests using lower concentrations of test substance (in the region of 1 to 100 µg/l) than the RBT, and specific chemical

analysis or radiolabelling techniques to measure biodegradation, which considerably increase the cost of biodegradation testing. The simulation tests may be subdivided according to the environmental compartment which they are designed to reflect; activated sludge (OECD, 2001), soil (OECD, 2002b), aquatic sediment (OECD, 2002a), and surface water (OECD, 2004). Supplementary information regarding the biodegradation of a substance under optimised conditions may also be investigated, if desired, by testing for inherent biodegradability, which will indicate whether or not a chemical has any potential for degradation under aerobic conditions. The more sophisticated nature of the simulation and inherent biodegradation tests means that degradation rates or half-lives may be estimated from the data, but this is not the case for the RBT.

1.3.1 The Ready Biodegradation Test (RBT)

The RBTs are a series of tests designed to assess the ease with which a chemical may be degraded by microorganisms ubiquitous in the environment (Vazquez-Rodriguez et al., 2007) and positive results are treated as unequivocal, i.e. it is assumed that the test compound will undergo rapid biodegradation in the environment. Generally, test chemicals are dissolved or suspended in a defined mineral medium and incubated at 22°C in the dark, or in diffuse light, in the presence of a bacterial inoculum over a 28 day period. Biodegradation is generally detected by non-specific parameters such as the removal of dissolved organic carbon (DOC); evolution of CO_2, or measuring the biochemical oxygen demand (BOD) in the test system (OECD, 1992; Reuschenbach et al., 2003). It is therefore necessary to run parallel 'blank' tests, containing no test substance, to account for endogenous degradative activity within the inoculum. Samples are deemed to be 'readily biodegradable' if they achieve a level of 60% or 70% biodegradation (depending on the method used; Table 1.2) in a 10 day period following the onset of biodegradation, which is deemed to commence at 10% biodegradation, all within a 28 day limit (Figure 1.1). The RBT also allows for different pre-treatments of inocula prior to use for testing. Inoculum pre-treatment is principally used to lower blank values and is sometimes necessary due to the non-specific methods used to monitoring test compound degradation; i.e. the amount of organic carbon added to the test system in the inoculum may exceed that added as test compound and so it is desirable to remove this carbon prior to testing.

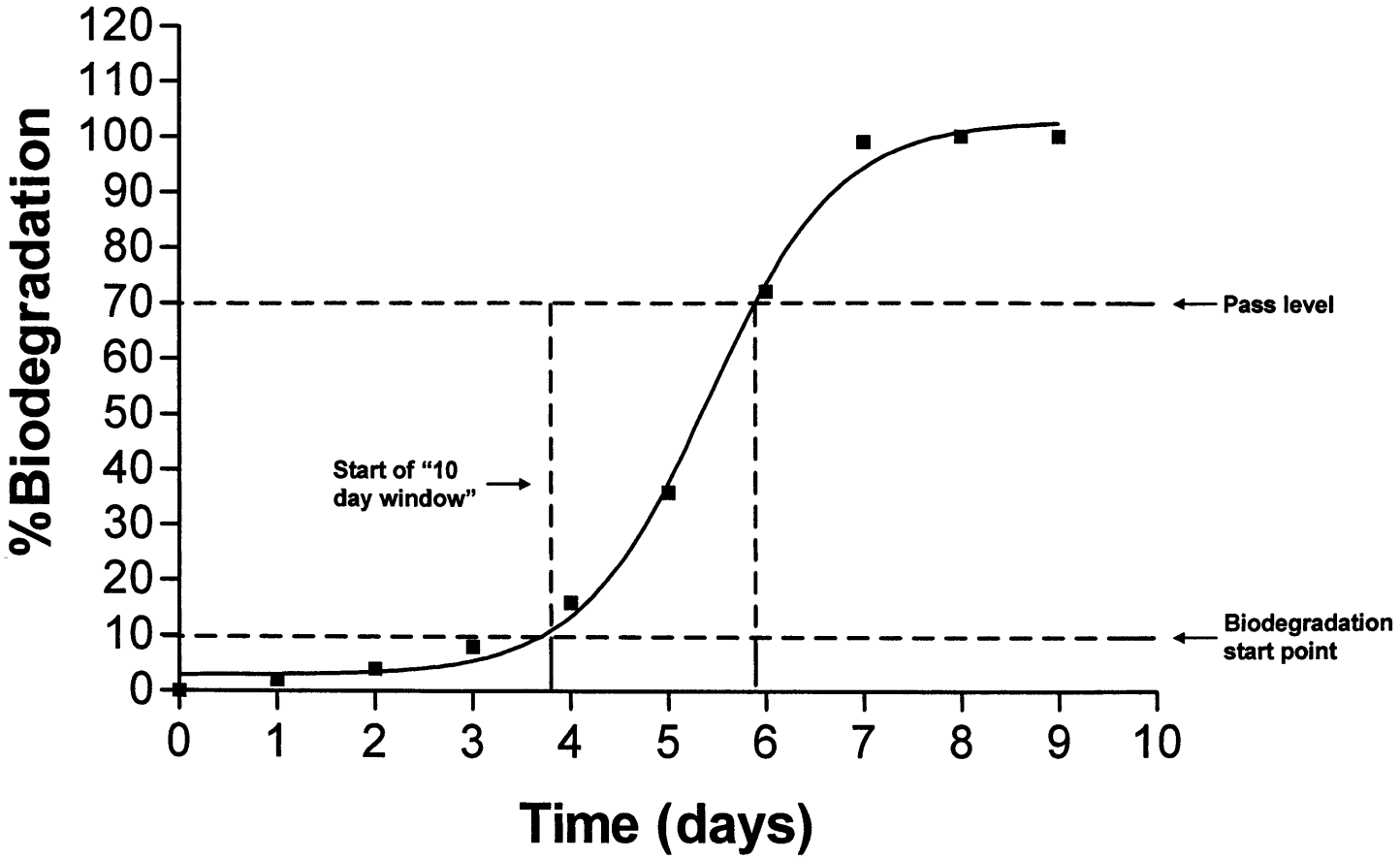

Figure 1.1 RBT parameters. Test chemicals are deemed to be readily degradable if they achieve the pass level (60 or 70%, depending on the method used) within a 10 day period, termed the "10 day window", following the onset of biodegradation, which is deemed to begin at 10% biodegradation. Samples failing to achieve the pass level within the 28 day test limit require further testing to determine their biodegradability.

1.3.2 Reproducibility of the RBT

RBTs suffer from a need to maintain both experimental reproducibility and environmental realism. Ingerslev et al. (2000) proposed that simpler systems, containing fewer variables (e.g. pure cultures) would be preferable, even though they would not reflect accurately the environmental fate of chemicals. Currently used RBTs are more complex systems which suffer from poor reproducibility. Explanations for this lack of reproducibility include differences in the concentration of the test chemical used (Ahtiainen et al., 2003) and differing test volumes (Ingerslev et al., 2000). However, the lack of regulation regarding the inoculum origin and pre-treatment is likely the principal reason for the inconsistent RBT results reported (Thouand et al., 1995; Vazquez-Rodriguez et al., 2007). The source of inoculum for the RBTs is not defined in detail, and a range of different inocula such as surface waters, soil and activated sludge are all permitted (Forney et al., 2001; Paixão et al., 2006; Vazquez-Rodriguez et al., 2007).

Table 1.2 Pass levels for different RBTs

Paramater measured	RBTs					
	DOC[a] die-away	CO_2 evolution	Manometric respirometry	Modified OECD screening	Closed bottle	MITI (I)
% DOC removal	70			70		
% ThOD[b]			60		60	60
% $ThCO_2$[c]		60				

a. DOC – dissolved organic carbon; b. ThOD – theoretical oxygen demand; c. $ThCO_2$ – theoretical CO_2 production.

1.3.3 Potential improvement of the RBT

Several methods of improving the reproducibility of the current RBTs have been described. van Ginkel et al. (1995) described improved degradation of diethylene glycol and diethylenetriamine in biodegradation tests following inoculum acclimation to the test chemical, though pre-exposure of RBT inocula to the test chemical is not allowable according to OECD guidelines. Vásquez-Rodríguez et al. (2007) studied the degradation potential of several activated sludges, following a standard 7 day pre-conditioning period, where the inoculum is incubated at test temperature and in test

medium with no exogenous carbon source. Their findings suggested that whilst pre-conditioned inocula gave more reproducible biodegradation kinetics for RBTs using acetate as the test substance, they lost their ability to degrade aniline – which is a known readily degradable xenobiotic. Other studies have shown some success using defined mixtures of organisms as substitutes for activated sludge, however they highlight the need for further testing such methods with a broader range of compounds to fully understand their potential as activated sludge surrogates (Paixão et al., 2006).

1.4 HALOGENATED ORGANIC COMPOUNDS AS XENOBIOTICS

Despite dominating EU listings of dangerous substances (e.g. Directive 2008/105/EC) halogenated organic compounds have been used extensively as herbicides, pesticides, preservatives and solvents, as well as for other applications. Their widespread use has lead to their extensive release into the environment, where they are considered xenobiotic, and raises concerns regarding the persistence and toxicity of many of these compounds. However, the commonly held belief that the majority of halogenated organics found in the environment are of anthropogenic origin has been called into question; as many as 3,800 organohalogens are now known to occur biogenically, or from natural abiogenic processes such as forest fires, volcanoes and other geothermal processes (Gribble, 2003). Indeed, even the highly toxic and reactive substance vinyl chloride, which was thought to be exclusively man-made, is now known to be formed naturally (Keppler et al., 2002). The biogenic formation of many organohalogens, such as the glycopeptide antibiotic vincomycin, and the antifungal pyrrolnitrin, is catalysed by microbial halogenating enzymes (Hammer et al., 1997; Williams and Bardsley, 1999). It has been proposed that chlorine participates in an extensive biogeochemical cycle, driven in part by the microbial production, incorporation and removal of chlorine in the natural environment (Öberg, 2002).

1.4.1 Toxicity of halogenated organic compounds

Halogenated xenobiotic compounds are notorious because of their detrimental effect on the environment and human health, owing largely to their acute and chronic toxicity, their bioaccumulation, and their persistent nature (Smidt and de Vos, 2004). Following an industrial accident in the Italian town of Seveso, significant quantities of

a the dioxin, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), were released into the surrounding area, and have since been linked with increased mortality from cardiovascular and respiratory diseases as well as various forms of cancer (Bertazzi et al., 1998). Dichlorodiphenyltrichloroethane (DDT), applied extensively as an insecticide to combat the malaria causing mosquito, is also well documented as being highly toxic and bioaccumulative, whilst other chlorinated xenobiotics such as polychlorinated biphenyls (PCBs) and several other chlorinated pesticides are known to adversely affect foetal development (Crinnion, 2009).

It is well known that the presence of halogenated compounds can significantly affect cellular metabolic processes, commonly due to the toxic effects of halogenated analogues of intermediary metabolites (Slater et al., 1995). A good example of this is the toxic effect of fluoroacetate following its conversion to fluorocitrate, a potent inhibitor of the Krebs cycle enzyme, aconitase (Peters, 1952). The α-halocarboxylic acid (αHA) monochloroacetic acid (MCA), historically applied as a herbicide, has been linked to the inhibition of liver gluconeogenesis by inactivating glyceraldehyde-3-phosphate dehydrogenase (Sakai et al., 2005), whilst iodoacetic acid is a known protein inhibitor owing to its ability to affect the sulphydryl groups of cystein residues (Carne et al., 1976). The removal of the halogen moiety by microbial degraders can relieve some of these inhibitory effects, providing alternative sources of carbon and energy for growth (Slater et al., 1997), as well as reducing recalcitrance to biodegradation (Janssen et al., 2001).

1.5 Microbial Dehalogenation

Dehalogenation is the process of cleaving the carbon halogen bond, and the release of halide ions. This may be achieved abiotically, or in a biologically catalysed reaction involving microbial enzymes termed dehalogenases. The first enzymes to show dehalogenating activity were identified by Jensen (1957a; 1957b) who coined the term dehalogenase. Currently, the term dehalogenase encompasses an enormous diversity of catabolic enzymes, with a range of substrate specificities and mechanisms of action. Dehalogenases have historically been classified according to their mechanism of action (Fetzner and Lingens, 1994; Janssen et al., 1994), though this method of classification has more recently been augmented by molecular phylogenetic methods (Janssen et al., 1994; Slater et al., 1997; Hill et al., 1999), and

the increasing number of crystallised structures (Verschueren et al., 1993; Hisano et al., 1996; Ridder et al., 1997; Li et al., 1998; Newman et al., 1999; Ridder et al., 1999; Marek et al., 2000; Schmidberger et al., 2005; 2008) for dehalogenases gives broader insight into their mechanisms of action.

1.5.1 Oxygenolytic dehalogenation

Oxygenolytic dehalogenation is carried out by mono or dioxygenases and results in the incorporation of molecular oxygen into the substrate. *Pseudomonas* sp. CBS3 is able to perform the oxygenolytic dehalogenation of 4-chlorophenylacetate to form 3,4-dihydroxyphenylacetate, via a direct oxidative attack of the aromatic ring by a two component 3,4-dioxygenase system which requires NADH (Markus et al., 1984; 1986). *Sphingomonas paucimobilis* UT26 contains the 1,2-dioxygenase LinE, responsible for the meta cleavage of chlorohydroquinone (CHQ) resulting in substrate dechlorination to form maleylacetate during γ-hexachlorocyclohexane (γ-HCH or lindane) degradation (Miyauchi et al., 1999). Interestingly, total mineralisation of γ-HCH requires several different dehalogenases which are discussed below (also, see Figure 1.2).

1.5.2 Dehydrohalogenation

Dehydrohalogenation is a mechanism involving the elimination of HCl from the parent molecule, leading to the formation of a double bond. LinA from *S. paucimobilis* UT26 catalyses the first two dehydrochlorination steps of γ-HCH degradation (Figure 1.2) (Nagata et al., 1999). LinA is distinct from the two previously identified dehydrochlorinases (Lipke and Kearns, 1959; Nagasawa et al., 1982) as it requires no cofactors for its activity.

1.5.3 Dehalogenation by methyl transfer

Many organisms are capable of degrading halogenated single carbon compounds such as methyl chloride or methyl bromide (McDonald et al., 2002). Most previously identified bacteria capable of methyl halide degradation are aerobic; e.g. the methylotrophic bacterium *Methylobacterium* sp. CM4 which dehalogenates chloromethane to tetrahydrofolate via two methyltransferase enzymes, CmuA (which has separate methyltransferase and corrinoid binding domains) and CmuB, releasing Cl^- as HCl (Vannelli et al., 1999). Similar dehalogenating systems have also been

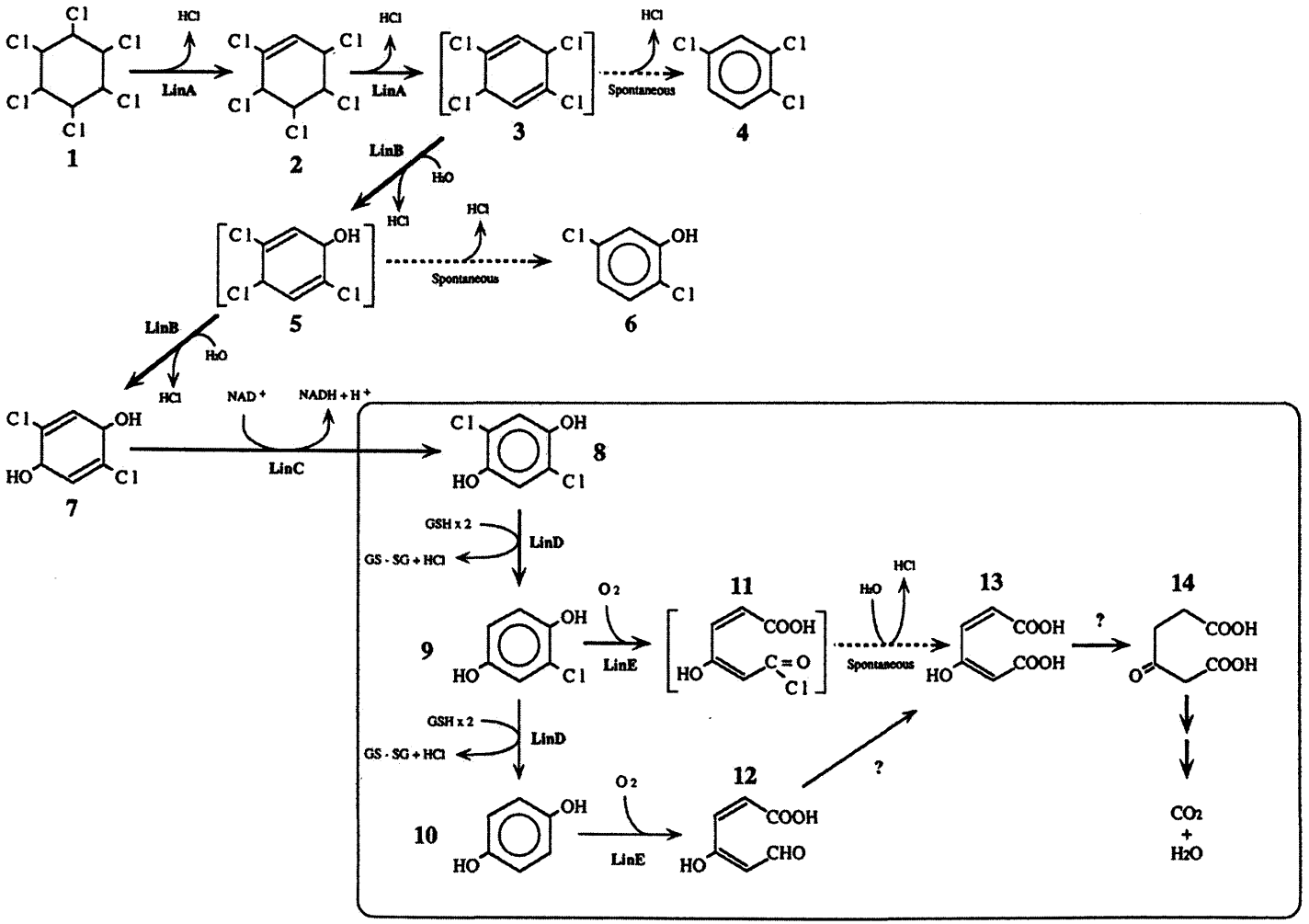

Figure 1.2 Proposed biodegradation pathway for γ-HCH in *S. paucimobilis* UT26. Compounds: 1 = γ-HCH; 2 = γ-pentachlorocyclohexane; 3 = 1,3,4,6-tetrachloro-1,4-cyclohexadiene; 4 = 1,2,4-trichlorobenzene; 5 = 2,4,5-trichloro-2,5-cyclohexadiene-1-ol; 6 = 2,5-dichlorophenol; 7 = 2,5-dichloro-2,5-cyclohexadiene-1,4-diol; 8 = 2,5-dichlorohydroquinone; 9 = chlorohydroquinone; 10 = hydroquinone; 11 = acylchloride; 12 = γ-hydroxymuconic semialdehyde; 13 = maleylacetate; 14 = β-ketoadipate (Miyauchi et al., 1999).

proposed for *Hyphomicrobium chloromethanicum* CM2 (McAnulla et al., 2001) and two *Alphaproteobacteria*, strains IBM-1 (Woodall et al., 2001) and CC495 (Coulter et al., 1999). The strictly anaerobic organism *Acetobacterium dehalogenans* (Meßmer et al., 1993) also dechlorinates chloromethane in a methyltransferase catalysed reaction (Wohlfarth and Diekert, 1997). Halogenated C1 compounds may also be degraded cometabolically by some methanotrophic, nitrifying and ammonia-oxidising bacteria (Rasche et al., 1990; Duddleston et al., 2000; Han and Semrau, 2000).

1.5.4 Reductive dehalogenation

Reductive dehalogenation is an important process in the degradation of many of the most persistent pollutants, including highly chlorinated polychlorinated biphenyls (PCBs), tetrachloroethene (PCE) and pentachlorophenol (PCP) (Mohn and Tiedje, 1992). Reductive dehalogenation occurs mainly anaerobically in a process known as dehalorespiration, though it is also known to occur under aerobic conditions (Criddle et al., 1986).

1.5.4.1 Glutathione S-transferase mediated reductive dehalogenation

Aerobic reductive dehalogenation may be accomplished by the glutathione S-transferase PcpC from *Flavobacterium* sp. strain ATCC 39723, which converts tetrachlorohydroquinone (TCHQ) to trichloro- and then dichlorohydroquinone using glutathione as the reducing agent (Orser et al., 1993). A halogen is first displaced from TCHQ through substitution with glutathione, which is then its self displaced from the aromatic ring by another glutathione molecule, producing oxidised glutathione (Xun et al., 1992). LinD is a glutathione S-transferase from *S. paucimobilis* UT26 which catalyses the dehalogenation of 2,5-dichlorohydroquinone to CHQ, and CHQ to hydroquinone (Figure 1.2) during the degradation of γ-HCH (Miyauchi et al., 1998).

1.5.4.2 Dehalorespiration

Dehalorespiration is a process where bacteria use halogenated organic compounds as the terminal electron acceptor during anaerobic respiration. Dehalorespiration was first reported in *Desulfomonile tiedjei* DCB-1, and since then several other examples of dehalorespiring bacteria have been identified, principally from the *Desulfitobacterium* and *Dehalococcoides* genera (Futagami et al., 2008).

Dehalorespiration has become of increasing interest due to the ineffective degradation of multiply halogenated xenobiotics via aerobic mechanisms (Furukawa, 2006).

Dehalorespiring bacteria have been reported to be capable of degrading several compounds, including PCE, trichloroethene, PCBs, hexachlorobenzene and PCP, amongst others (Mohn and Tiedje, 1992; Furukawa, 2006; Futagami et al., 2008). Until recently, there were no reports of dehalorespiring bacteria utilizing α-halocarboxylic acids (αHAs); however, De Wever et al. (2000) identified a bacterium capable of the anaerobic reductive dehalogenation of trichloroacetic acid (TCA).

1.5.5 Hydrolytic dehalogenases

One large group of dehalogenases which have received much attention are the hydrolytic dehalogenases. These enzymes catalyse a nucleophilic substitution which requires only water as a cofactor, and include the haloalkane dehalogenases and the αHA dehalogenases (Janssen et al., 1994).

1.5.5.1 Haloalkane dehalogenases

One of the best studied dehalogenases to date is DhlA of *Xanthobacter autotrophicus* GJ10, which is a soluble protein 310 amino acids long showing activity on a range of haloalkanes (Janssen et al., 1989). The reaction mechanism was determined by x-ray crystallography to show that dechlorination of 1,2-dichloroethane is achieved via a two step catalytic mechanism; Asp_{124} acts as a nucleophile, attacking the substrate and forming a covalently bound ester intermediate which is subsequently cleaved by an activated water molecule (Verschueren et al., 1993; Fetzner and Lingens, 1994). The structures of two other haloalkane dehalogenases have also been identified – DhaA from a *Rhodococcus erythropolis* (Newman et al., 1999) and LinB from *S. paucimobilis* UT26 (Marek et al., 2000). LinB has been shown to catalyse the third and fourth steps of γ-HCH degradation (Figure 1.2; Nagata et al., 1993). The dehalogenase DehH1, encoded by *dehH1* on plasmid pUO1 of *Delftia acidovorans* strain B (formerly *Moraxella* sp. strain B) was originally identified for its activity against fluoroacetic acid, though sequence analysis has since shown that this gene is similar to several haloalkane dehalogenases (Kawasaki et al., 1992).

More recently, genomic analysis – where genome sequences are searched for haloalkane dehalogenase like sequences – has allowed the identified several putative haloalkane dehalogenases from organisms not previously associated with dehalogenase activity, including the human pathogen *Mycobacterium tuberculosis* H37Rv (Jesenská et al., 2000) and other *Mycobacteria* (Jesenská et al., 2000; 2002), *Mesorhizobium loti* MAFF303099 and *Bradyrhizobium japonicum* USDA110 (Sato et al., 2005).

1.5.5.2 α-Halocarboxylic acid (αHA) dehalogenases

The αHA dehalogenases have also been intensively studied providing an abundance of information regarding their phylogenetic relationships, structures, and mechanisms of action. This group consist of two evolutionary and functionally distinct enzyme families; the group I (*dehI*) and group II (*dehII*) αHA dehalogenases (Figure 1.3; Hill et al., 1999). The group I enzymes are phylogenetically distinct from group II and have no close evolutionary relatives. Group I dehalogenases generally show broader stereoselectivity than the group IIs, with activity against both D- and L-haloacids, although some, such as *dehI*° of *Pseudomonas putida* PP3 and *hadD* of *P. putida* AJ1, show activity with D-haloacids only (Barth et al., 1992; Hill et al., 1999). Interestingly, *dehI* subgroups C and D (Figure 1.3A) contain only dehalogenases acting on D-haloacids, whilst *dehI* subgroups A and B (Figure 1.3A) contain dehalogenases with activity against both D- and L- haloacids.

Dehalogenation by group I enzymes involves a direct attack of an enzymatically activated water molecule on the C2 carbon of the haloacid, displacing the halogen atom (Nardi-Dei et al., 1999), as illustrated in Figure 1.4A. No covalently bound enzyme-substrate intermediate is formed (as seen with the haloalkane and group II haloacid dehalogenases). Recently, the first crystal structure of a group I dehalogenase, DehI from *P. putida* PP3, was solved (Schmidberger et al., 2008). As well as providing valuable insight into the stereoselectivity of this D-/L- active

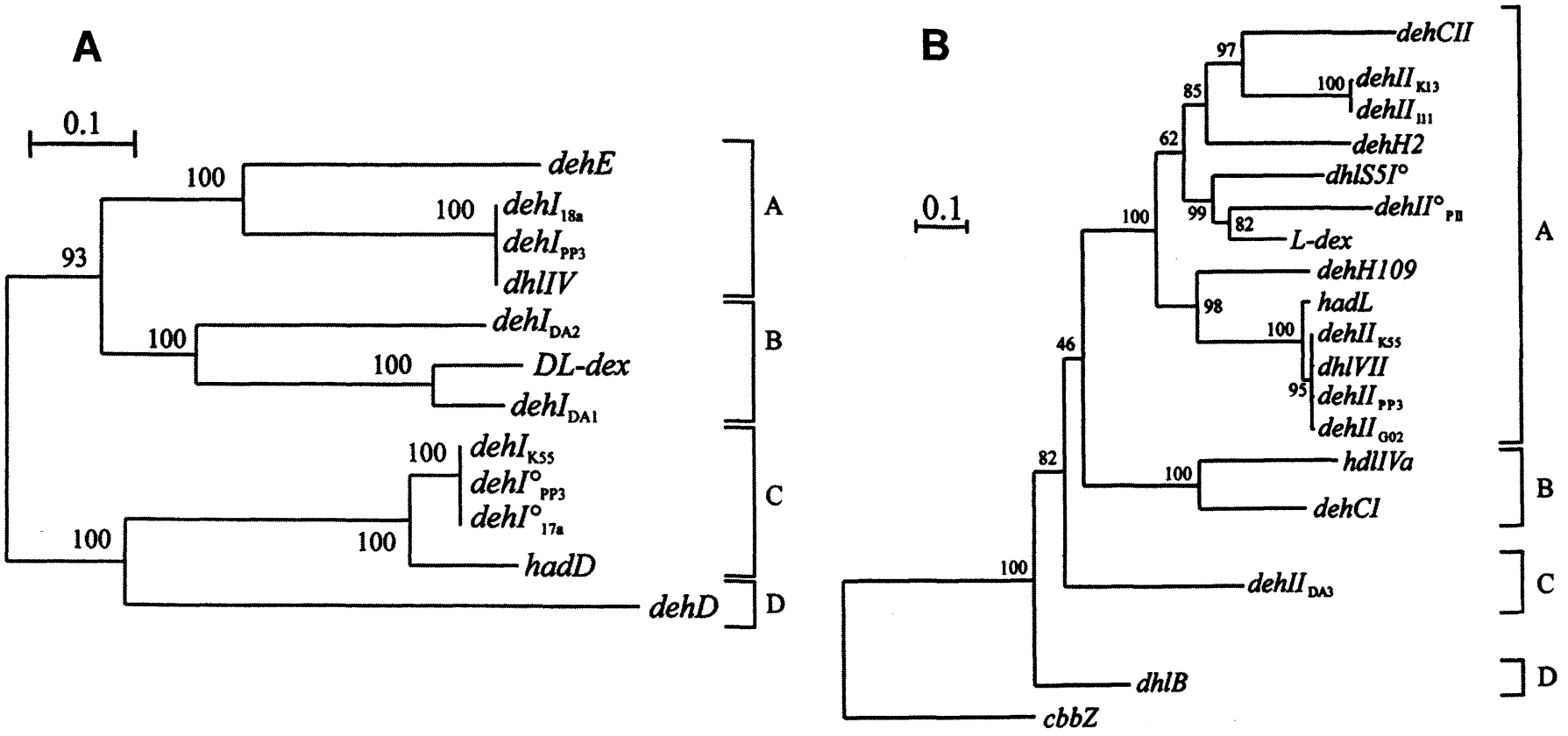

Figure 1.3 Dendrogram illustrating the relationship between previously characterised αHA dehalogenase genes. A) group I dehalogenases (*dehI*); B) group II dehalogenases (*dehII*) and *cbbZ*, a 2 phosphoglycolate phosphatase from *Alcaligenes eutrophus*, which is a member of the HAD gene superfamily. Reproduced from (Hill et al., 1999).

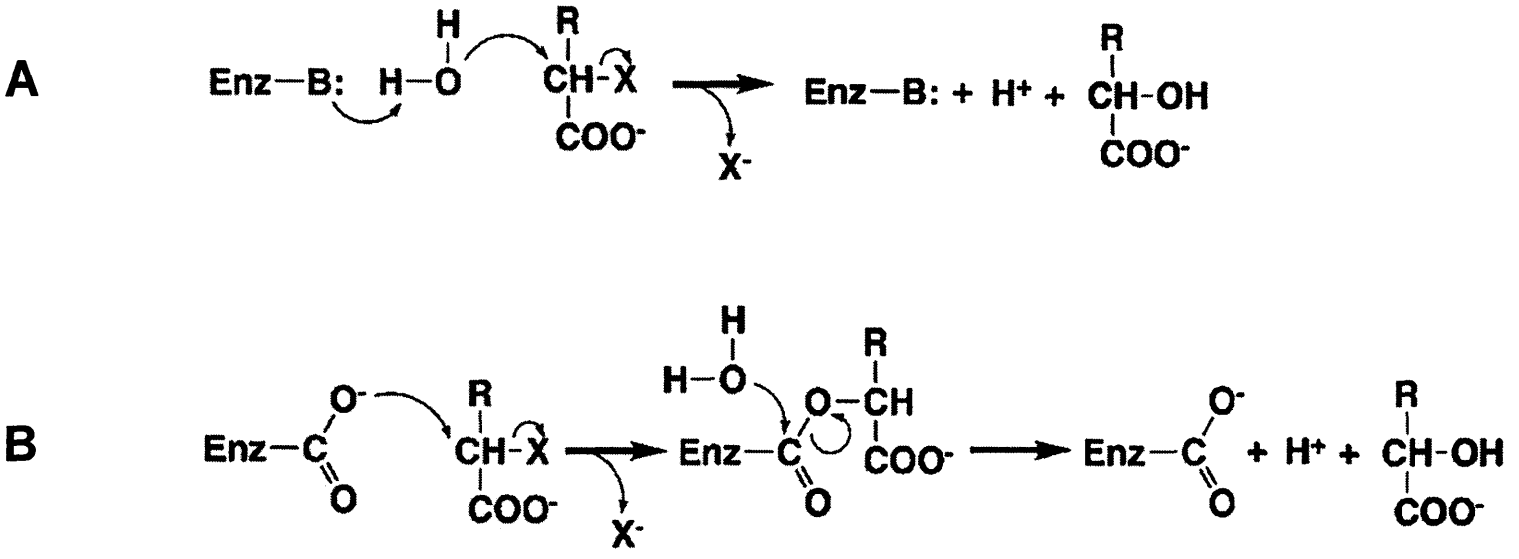

Figure 1.4 Reaction mechanism for DehI and DehII enzymes. (A) For DehI enzymes, a general base – B: – activates a water molecule which attacks the halogenated compound, displacing the halogen. (B) For DehII enzymes, the halogenated compound undergoes a nucleophilic attack by an active site carboxylate group, resulting in halogen removal and the formation of an ester intermediate, which is then hydrolysed by an enzyme activated water molecule. X represents a halogen atom, R represents a hydrogen atom or an alkyl group. Reproduced from (Nardi-Dei et al., 1999).

enzyme, this study also suggested that the selectivity of the D- only enzymes is due to the substitution of a single amino acid residue (Ala187Asn) preventing the productive binding of L-haloacids (Schmidberger et al., 2008).

The group II enzymes (Figure 1.3B) constitute a large clade within the haloacid dehalogenase (HAD) superfamily (Koonin and Tatusov, 1994), a broad grouping of hydrolytic enzymes spanning the bacterial, eukaryal and archaeal kingdoms, which includes the epoxide hydrolases, magnesium dependent phosphatases and P-type ATPases (de Jong and Dijkstra, 2003). Group II dehalogenases show activity exclusively with L-haloacids. Dehalogenation is achieved by a nucleophilic attack of the haloacid C2 carbon by an aspartate residue, forming an esterified enzyme-substrate intermediate which is subsequently cleaved by a second nucleophilic attack by an activated water molecule targeting the aspartate C2 carbon, yielding the free enzyme and product (Figure 1.4B; Li et al., 1998; Ridder et al., 1999). A similar reaction mechanism, involving covalently bound enzyme-substrate intermediates, is found in several haloalkane dehalogenases (Janssen, 2004) – although they share no significant similarity to the αHA dehalogenases at the nucleotide sequence level (van der Ploeg et al., 1991) – and the 4-chlorobenzoyl-CoA dehalogenase of *Pseudomonas* sp. CBS3 (Yang et al., 1994; Benning et al., 1996).

1.6 Genetics of Dehalogenases and Dehalogenating Organisms

Bacterial genes encoding related functions, such as catabolic genes and their associated regulatory and transport genes, are often clustered within the bacterial genome. Gene clusters may be selected for due to the benefits of co-regulation of all genes related to a similar function; closely clustered genes may be co-transcribed as a unit referred to as an operon (Lawrence, 2002). Their organisation may also reflect a selfish property of the genes themselves, where by their fitness (not necessarily the fitness of the organism as a whole) is benefited due to the increased likelihood of their co-transfer to another genome via horizontal gene transfer (HGT) (Lawrence and Roth, 1996). HGT is a well documented phenomenon affecting bacterial evolution and adaptation, the driving units of which are mobile genetic elements (MGE) such as plasmids, insertion sequences, integrons and transposons. HGT is most commonly associated with the spread of antibiotic resistance genes following the widespread use of antibiotics (Davies, 1994), although it is also a driving force in the adaptation of

bacteria to xenobiotic contaminated environments, either by increasing the number of organisms within a community which are able to degrade these compounds, or by generating new combinations of existing genes to form novel catabolic pathways (Top et al., 2002).

There are many well characterised examples of xenobiotic catabolic gene clusters carried by MGE. Many catabolic genes are carried on self transmissible plasmids, such as the atrazine catabolic plasmid pADP1 of *Pseudomonas* sp. strain ADP, which carries the complete atrazine degradation pathway, including genes *atzA, atzB* and *atzC* at disparate locations which are themselves closely associated with IS*1071*-like insertion sequences, as well as the genes *atzDEF* which are co-transcribed (Martinez et al., 2001). Genomic islands (GI) are a group of MGE related to the so called integrative and conjugative elements (ICE) which commonly contain genes related to pathogenicity (Burrus et al., 2002). The *clc* element of *Pseudomonas* sp. strain B13 is a GI, or integron, best known for conferring on its host the ability to degrade chloroaromatic compounds (Gaillard et al., 2006). This element uses a bacteriophage like integrase to excise and reintegrate site specifically into the glycine tRNA gene, thus allowing the horizontal transfer of the entire 105 kbp element (Top et al., 2002; Gaillard et al., 2006). Both class I and class II transposons have been shown to carry catabolic genes or gene clusters: class I transposons, or composite transposons, consist of two identical or nearly identical insertion sequences (IS) flanking centrally located (catabolic) genes; where as class II elements are associated with short terminal inverted repeats and undergo replicative transposition involving transposase and resolvas enzymes (Top et al., 2002). The genes for chlorobenzoate degradation from *Alcaligenes* sp. BR60 are carried on the class I catabolic transposon Tn*5271*, which is its self situated on the plasmid pBRC60 (Nakatsu et al., 1991), whilst the class II transposons Tn*4651* (Tsuda and Iino, 1987) and Tn*4653* (Tsuda and Iino, 1988) carry the toluene / xylene degradation genes within plasmid pWW0.

Gene clustering and/or association with MGE is also common to dehalogenase genes. *P. putida* PP3 contains a catabolic gene cluster consisting of the haloacid dehalogenase gene *dehII* and its regulator *dehR*, along with the putative permease gene *dehP*, as well as the silent *dehI°* gene (Figure 1.5). Dodds (2003) suggested that

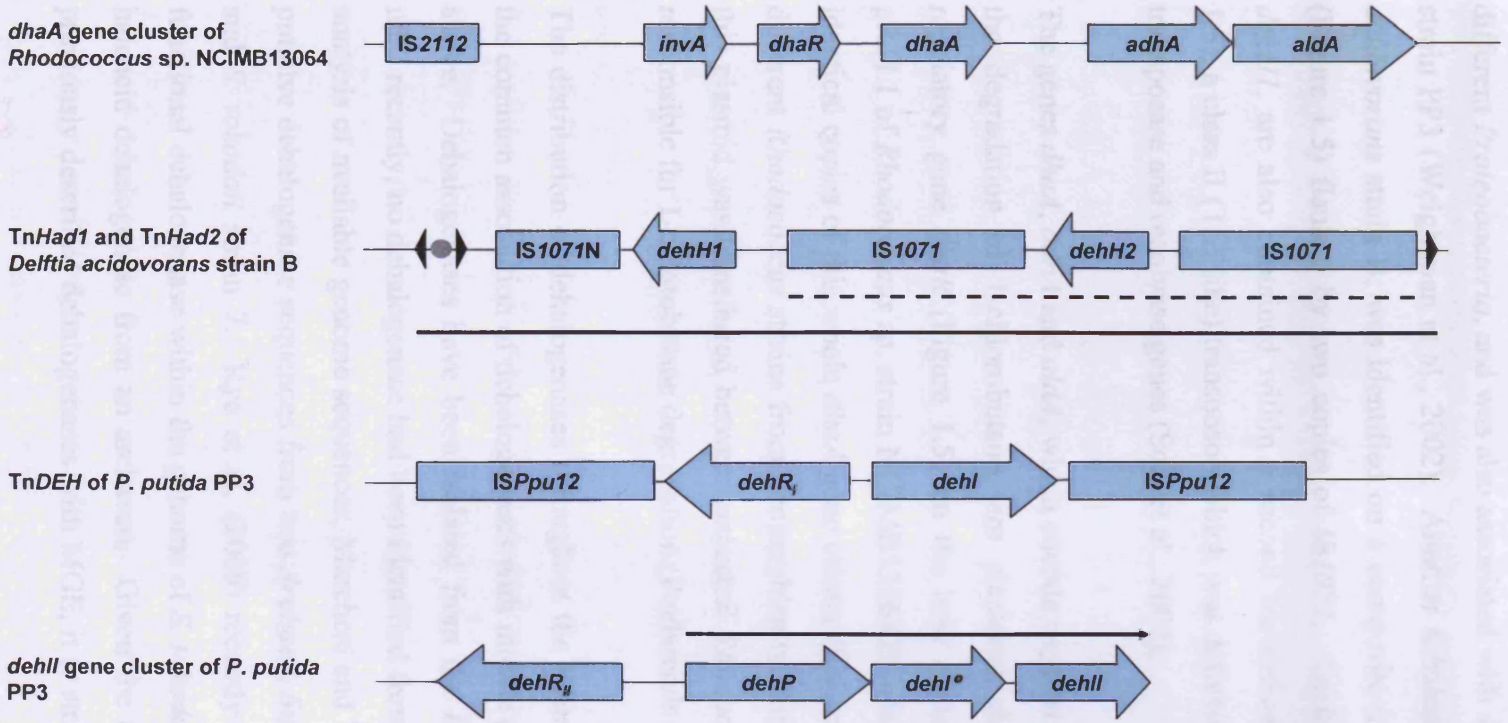

Figure 1.5 Clustering of bacterial dehalogenases and associated regulatory genes, and their associations with MGE. Blocked arows represent open reading frames. Blocks represent insertion sequences. *Rhodococcus* sp. strain NCIMB13064 contains the haloalkane degradative genes *dha*, *adhA* and *aldA*, the regulatory gene *dhaR*, the putative invertase gene *invA*, and insertion element IS*2112* (Poelarends et al., 2000b). *Delftia acidovorans* strain B contains the dehalogenase gene *dehH2* flanked by two copies of IS*1071* and *dehH1* which is adjacent to a truncated insertion sequence, IS*1071N* (Sota et al., 2002) – broken line indicates Tn*Had1*; solid line indicates Tn*Had2*; filled triangles represent inverted repeat sequences; filled circle represents a resolution (res) site. *P. putida* PP3 contains *dehI* and its regulator, $dehR_I$, flanked by two copies of insertion sequence IS*Ppu12*, making up Tn*DEH* (Weightman et al., 2002). *P. putida* PP3 also contains a three gene cluster encompassing the putative αHA transporter gene *dehP*, the silent *dehI°* and *dehII* genes, transcribed in the opposite orientation to the regulator gene, $dehR_{II}$ (Dodds, 2003) - black arrow represents the single mRNA transcript produced over this three gene cluster (Leggett and Weightman, unpublished).

the organisation of genes *dehP*, *dehI°* and *dehII* was reminiscent of a bacterial operon, which was experimentally confirmed recently using RT-PCR (M. J. Leggett and A. J. Weighman, Unpublished). Strain PP3 also contains a second dehalogenase gene cluster consisting of *dehI* and its cognate regulatory gene $dehI_R$ flanked by identical direct repeats designatied IS*Ppu12*. This structure was designated Tn*DEH* (Figure 1.5), a composite (class I) transposon 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* of *Delftia acidovorans* strain B, was identified on a composite transposon designated Tn*Had1* (Figure 1.5) flanked by two copies of IS*1071*. Tn*Had1*, along with dehalogenase *dehH1*, are also contained within a second transposon designated Tn*Had2* (Figure 1.5), a class II (Tn*3* like) transposon which was defective in transposition as it lacked transposase and resolvase genes (Sota et al., 2002).

The genes *dhaA*, *adhA* and *aldA*, which encode the first three enzymes responsible for the degradation of 1-chlorobutane, are clustered along with the putative *dhaA* regulatory gene *dhaR* (Figure 1.5) on the host chromosome, and on the plasmid pRTL1 of *Rhodococcus* sp. strain NCIMB13064 (Poelarends et al., 2000a). Virtually identical copies of this whole *dhaA* gene cluster (Figure 1.5) were detected in several different *Rhodococcus* strains from geographically distinct locations, suggesting that this plasmid was transferred between ancestral *Rhodococci*, disseminating the genes responsible for 1-chlorobutane degradation (Poelarends et al., 2000b).

The distribution of dehalogenases throughout the bacterial kingdom is likely due to the common association of dehalogenases with mobile genetic elements, as discussed above. Dehalogenases have been isolated from the *Eukarya* and *Bacteria*, though until recently, no dehalogenase had been identified from the *Archaea*. Using *in silico* analysis of available genome sequences, Marchesi and Weightman (2003b) identified putative dehalogenase sequences from two *Archaea*, *Sulfolobus solfataricus* strain P2 and *S. tokodaii* strain 7. Rye et al. (2009) recently confirmed the presence of a functional dehalogenase within the genome of *S. tokodaii* strain 7, the first confirmed haloacid dehalogenase from an archaeon. Given the common association of many previously described dehalogenases with MGE, it is striking that αHA dehalogenases

are confined almost exclusively to the *Proteobacteria*. Although several studies have identifying Gram positive organisms capable of degrading αHA (Kearney et al., 1963; Kerr and Marchesi, 2006), only one of these studies reported data regarding the biochemistry of their dehalogenases; however no DNA sequence data was obtained (Chiba et al., 2009).

1.7 Molecular Genetic and Cultivation-Independent Investigations of Microbial Communities

1.7.1 The great plate count anomaly

Microbial life was discovered little over 300 years ago with the invention of Leeuwenhoek's microscope, though the simple morphologies of most microbial cells prevented their classification by the morphological features traditionally used for higher organisms (Pace, 1997). The advent of pure culture studies broadened our understanding of the microbial world, but it is now known that laboratory cultivation gives a very narrow view of microbial diversity, as the majority of bacteria are uncultivable using standard methods (Wagner et al., 1993). It has been frequently reported that direct microscopic cell counts exceeded viable cell counts, estimated by plate counting, by several orders of magnitude (e.g. Amann et al., 1995). Staley and Konopka (1985) termed this phenomenon the "great plate count anomaly". The discrepancy between the total bacterial population and the cultivable population therein is particularly pronounced in soils, where only 0.1-1% of bacteria are cultivable using standard laboratory conditions (Torsvik et al., 1990; Torsvik and Øvreås, 2002).

1.7.2 rRNA methods for the cultivation-independent phylogenetic identification of bacteria

The ribosomal RNA (rRNA) sequence analysis approach developed by Carl Woese revolutionised the classification not only of microbes, but of all organisms, allowing the construction of a tree of life, and provided a powerful tool with which to study this uncultivated majority (Woese, 1987). Woese described rRNA genes as 'the ultimate molecular chronometers', or molecular clocks, due to their functional consistency within all organisms and their highly conserved structures and sequences. There are three types of prokaryote rRNA, classified according to their sedimentation rates during ultracentrifugation as 23S, 16S and 5S, with sequence lengths of

approximately 3300, 1650 and 120 nucleotides, respectively (Rosselló-Mora and Amann, 2001). The relatively short sequence length of the 5S rRNA gene provided little phylogenetic information, and whilst the 23S rRNA gene sequence was the largest of the three genes, the 16S rRNA gene became the standard marker for bacterial classification because it can be sequenced (from DNA and cDNA templates) more easily and rapidly (Spiegelman et al., 2005). The 16S rRNA forms a structural component of the 30S ribosomal small subunit, and consists of eight highly conserved regions (U-U8), and nine variable regions (V1-V9), which are, presumably, less important for ribosome function (Jonasson et al., 2002). The presence of both highly conserved and highly variable regions with different evolutionary rates within the 16S rRNA gene, combined with their apparent lack of interspecies horizontal gene transfer (HGT) (Woese et al., 1980; Olsen et al., 1986) also makes them excellent chronometers (Jonasson et al., 2002). Genes which are prone to HGT can not be used as chronometers as their evolution is not intrinsically linked to the host organism, only to the gene itself (Woese, 1987). Today, many different techniques have been developed with which to study rRNA sequences, which continue to be the gold standard for microbial classification, as well as the basis of most studies investigating microbial diversity in the natural environment.

1.7.3 Molecular systematics and the development of cultivation-independent approaches to study microbial populations in the natural environment

Following advances in sequencing technology, rRNA based methods began to be employed not only for characterisation of single organisms, but for the identification of the complex makeup of entire populations. Early studies of community rRNA were conducted by preparing clone libraries of whole population DNA in bacteriophage lambda, which were subsequently screened by hybridisation with 16S rRNA gene specific probes, and selected for sequencing (Olsen et al., 1986). The advent of the polymerase chain reaction (PCR; Saiki et al., 1988) allowed the selective amplification of 16S rRNA gene sequences directly from mixed DNA templates, removing the need to laboriously screen for 16S rRNA containing clones (Amann et al., 1995; Theron and Cloete, 2000). Clone libraries produced from PCR amplification should contain only specific products which can be sequenced easily from known priming sites (Amann et al., 1995). This method was first used by Giovannoni et al. (1990) to study the microbial population of Sargasso Sea

picoplankton, and has been used extensively in the study of environmental bacterial populations since then (Mullins et al., 1995; Dabert et al., 2001; Juretschko et al., 2002).

The early studies of Giovannoni et al. (1990) and Mullins et al. (1995) identified the first examples of an uncultivated species, based entirely on the sequence of 16S rRNA genes, in the form of a distinct grouping within the *Alphaproteobacteria* with no close phylogenetic neighbours. This group, named the SAR11 cluster, had no cultivated representatives, and was one of the most abundant members of bacterioplankton communities in the subtropical oceans (Giovannoni et al., 1990; Mullins et al., 1995). The SAR 11 cluster was recognised purely on the basis of its 16S rRNA gene sequence until 2002, where the first members were cultivated and designated genus *Candidatus Pelagibacter* (Rappe et al., 2002).

1.7.4 DNA profiling methods for population studies: Denaturing gradient gel electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) is a method of separating DNA fragments based on their sequence composition (Fischer and Lerman, 1979). DNA fragments are run on a vertical polyacrylamide gel containing an increasing denaturing gradient of formamide and/or urea. An almost identical method, temperature gradient gel electrophoresis (TGGE), uses polyacrylamide gels with a denaturing temperature gradient in place of a chemical gradient. Fragments are separated when, on encountering sufficiently denaturing conditions, the helical form of the DNA fragment is melted, virtually halting further migration within the gel (Figure 1.6). The nucleotide composition of a DNA fragment determines its melting behaviour, and as such, the migration of fragments of differing nucleotide sequence will be halted at different locations within the gel (Theron and Cloete, 2000). The addition of a GC rich sequence (GC clamp) into one of the PCR primers prevents total fragment dissociation for optimal separation of DNA fragments by DGGE (Muyzer et al., 1993).

DGGE analysis of PCR amplified 16S rRNA gene sequences allows a simple and rapid assessment of microbial diversity within a population. As multiple samples may be examined concurrently on a single gel, DGGE is particularly well suited to

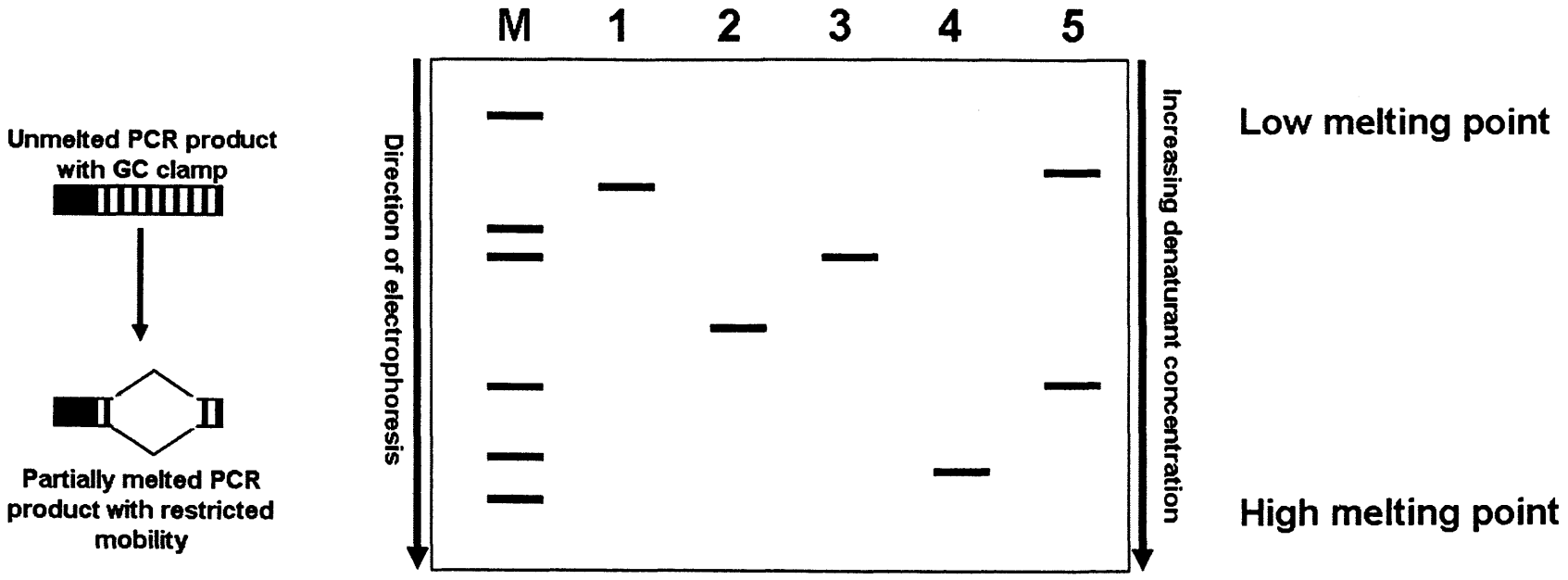

Figure 1.6 Mechanism of PCR product separation by DGGE. PCR products (e.g. 16S rRNA gene PCR products) are separated on a polyacrylamide gel with an increasing denaturing gradient. When the PCR product encounters sufficiently denaturing conditions, it melts, virtually halting its movement. Individual bands on the gel represent separate PCR products of differing sequence. M = a marker for inter gel comparison. Lanes 1-4 represent bacterial isolates with different 16S rRNA gene sequences, and therefore different mobility within the gel. 5 is a mixture of two different bacterial isolates.

examine temporal changes within the same population. The analysis of 16S rRNA genes by DGGE was first applied by Muyzer et al. (1993) and has since been used extensively to study bacterial, archaeal and eukaryotic diversity from a variety of environments, including; waste water treatment plants (Boon et al., 2002), fresh and salt waters (Casamayor et al., 2000; 2002), and soils (Gelsomino et al., 1999). D/TGGE has also been utilised for studying the diversity of genes other than 16S rRNA, including the *dsrB* gene from sulphate reducing populations (Geets et al., 2006) the *alkB* gene from crude oil amended soils (Hamamura et al., 2008), the *tfdA* gene from phenoxy acid herbicide degrading populations (Bælum et al., 2006), and the largest subunit of multicomponent phenol hydroxylases from activated sludge (Watanabe et al., 1998).

1.7.4.1 Other profiling methods

There are a variety of different PCR based techniques available for the study of microbial diversity. Single strand conformation polymorphism (SSCP), was first described by Orita et al. (1989). SSCP involves the separation of single stranded DNA fragments on a non denaturing polyacrylamide gel based on their three dimensional conformation, which is influenced by their sequence. This method has been used for the cultivation-independent identification of bacterial pathogens in blood cultures (Turenne et al., 2000) and various environmental samples (Schwieger and Tebbe, 1998; Smalla et al., 2007). Terminal restriction fragment length polymorphism (tRFLP), where fluorescently labelled, double stranded PCR products are subjected to a restriction digest allowing the visualisation of a terminal restriction fragment profile on an acrylamide gel or via capillary electrophoresis, was first described by Liu et al., (1997). Since then, tRFLP has been used for the characterisation of various bacterial communities (Clement et al., 1998; Miyamoto et al., 2004; Stralis-Pavese et al., 2006). These techniques both allow the assessment of species composition and diversity, and may also be applied to study the diversity of genes other than 16S rRNA (Junca and Pieper, 2004; Pester et al., 2004).

1.7.5 High throughput sequencing technology for community analysis

The scope of traditional 16S rRNA analysis, as outlined above, is limited in that only the dominant organisms present in an environment are detected, excluding the highly diverse, low abundance taxonomic groups termed "the rare biosphere" (Sogin et al.,

2006). This rare biosphere was virtually inaccessible using traditional cloning and Sanger sequencing technology, owing to its high cost and labour intensity. However, novel sequencing technology, the so called next generation sequencing or pyrosequencing, provides a method of producing vast quantities of sequence data and outperforms Sanger sequencing methods in terms of time taken and cost per nucleotide sequenced (Hugenholtz and Tyson, 2008).

Pyrosequencing involves the generation of DNA sequence using the sequencing by synthesis principle; each deoxyribonucleic acid triphosphate (dNTP) is individually added to the pyrosequencing reaction where, upon incorporation of a correctly matching nucleotide to the DNA template to be sequenced, a cascade reaction is triggered which ultimately releases a light signal indicating successful base pairing (Ronaghi et al., 1996). In this manner, the DNA template is directly sequenced as the reaction progresses. Margulies et al. (2005) described a highly parallel sequencing system where individual DNA fragments were bound to beads and suspended in an oil droplet containing PCR reagents. Following amplification, this resulted in beads carrying millions of copies of a unique DNA template, which were then sequenced using pyrosequensing technology. Significantly, this method does not require cloning of DNA fragments, removing associated biases and significantly reducing the labour intensity. This methodology has been widely utilised for the study of microbial diversity from many different environments, such as the human gut (Dethlefsen et al., 2008; Turnbaugh et al., 2009), soil (Roesch et al., 2007), and the deep marine biosphere (Sogin et al., 2006; Huber et al., 2007), where huge numbers of 16S rRNA gene variable regions (termed tags) are sequenced allowing researchers access to the rarer members of the community.

1.7.6 Whole cell fluorescent *in situ* hybridisation (FISH) methods

Whole cell hybridisation allows the identification of morphologically intact cells using labelled oligonucleotide probes targeting cellular rRNAs. This method has the advantage of being able to view cellular morphological features, as well as the spatial distribution of individual uncultivated organisms in situ (Amann et al., 1995). Using separate probes labelled with different fluorophores allows the identification of multiple taxonomic groups within a single sample, whilst counterstaining with 4',6-diamidino-2-phenylindole (DAPI) also enables total cell counts to be conducted

in the same samples (Amann et al., 1997). This technique has been applied to study the microbial community from several habitats, including activated sludge (Juretschko et al., 1998; 2002; Hesselsoe et al., 2009); microbial populations in biofilms (Manz et al., 1993; Ramsing et al., 1993; Daims et al., 2006) and marine environments (Cottrell and Kirchman, 2000; Gómez-Pereira et al., 2010).

1.7.7 Metagenomic approaches

Metagenomics is defined as the functional and sequence-based analysis of the collective microbial genomes contained in an environmental sample (Handelsman et al., 1998; Riesenfeld et al., 2004). The cloning of DNA directly from environmental samples was first described by Schmidt et al. (1991). Since then, the advancement of cloning and sequence technology has generated quantities of genomic information on a previously unimaginable scale (Riesenfeld et al., 2004). For instance, Venter et al. (2004) conducted the largest metagenomic study to date by sequencing the metagenome of the Sargasso sea, generating over 1 billion bp of sequence and identifying 148 previously unknown bacterial phylotypes and 1.2 million new genes. In contrast to studies which investigate microbial populations based on the 16S rRNA gene, metagenomic studies have identified novel functionality and activity such as antibiotic synthesis and resistance genes, as well as genes encoding degradative enzymes (Handelsman, 2004; Riesenfeld et al., 2004).

1.7.8 Stable isotope probing (SIP)

SIP is a cultivation-independent method allowing researchers to directly link a microorganism within a given environment to a specific metabolic activity. If a population is exposed to substrates labelled with stable isotopes, then the subsequent appearance of heavy labelled isotope within an organism's biomarker molecules (generally lipids or nucleic acids) indicates that it was involved in substrate assimilation (Kreuzer-Martin, 2007). This method may be applied using ^{13}C-labelled substrates to separate the nucleic acids of microorganisms actively involved in substrate assimilation (^{13}C-labelled DNA) from those not involved (^{12}C-DNA). Due to the different densities of ^{13}C- and ^{12}C-labelled DNA, these two fractions (representing functionally active and inactive populations, respectively) may be separated using CsCl density gradient centrifugation, and phylogenetically characterised using the PCR-based methods outlined above (Radajewski et al., 2000).

The use of ^{13}C-labelled substrates has been used to identify bacteria from various settings (Boschker et al., 2001; Radajewski et al., 2002; Hutchens et al., 2004), including the identification of pollutant assimilating bacteria (Liou et al., 2008). Stable isotopes other than ^{13}C (e.g. ^{15}N) have also been used for SIP analysis (Buckley et al., 2007).

1.7.9 Limitations of molecular genetic analysis

Molecular genetic studies of microbial populations have revolutionised our understanding of microbial ecology, overcoming the biases inherent in cultivation-dependent analysis. However, most molecular techniques have their own associated problems and biases, which are outlined below.

1.7.9.1 Nucleic acid extraction

Cell disruption and isolation of nucleic acids is the first step of any PCR-based approach and is a critical stage in the outcome of any study of microbial diversity. Preferential or sub-optimal disruption of some cell types would skew the observed community diversity, as cells resisting breakage will be under-represented, whilst those more prone to breakage would be over-represented in the resulting community profile; e.g. Gram negative cells are more susceptible to cell breakage than Gram positive cells (Theron and Cloete, 2000). However, the use of vigorous cell lysis procedures to extract nucleic acids from Gram positive cells may cause the fragmentation of nucleic acids from Gram negative cells. Fragmented nucleic acids significantly increase the probability of producing chimeric sequences during PCR (Wintzingerode et al., 1997) – see section 1.7.9.2 below. The smaller size of extracted rRNA molecules makes them less susceptible to fragmentation during mechanical cell disruption, making this a preferable target for amplification, via RT-PCR, though they are highly susceptible to nuclease catalysed hydrolysis during extraction (Wintzingerode et al., 1997; Theron and Cloete, 2000).

1.7.9.2 PCR of 16S rRNA genes

Amplification of community 16S rRNA or DNA by PCR (or RT-PCR) is a convenient and rapid method of studying bacterial diversity; however, PCR may introduce spurious sequences for a variety of reasons. Contaminating DNA is an obvious problem, where by additional variability is added to a sample from

exogenously introduced DNA, although this should present little problem with proper organisation and the use of appropriate decontamination measures, e.g. UV treatment of equipment and/or reagents prior to amplification (Sarkar and Sommer, 1990).

Another common artefact of PCR is the introduction of recombinant or chimeric sequences during amplification of highly fragmented or damaged templates (Paabo et al., 1990), or from very similar templates (Shuldiner et al., 1989). Ashelford et al. (2005) estimated that 5% of rRNA sequences available in the Ribosome Database Project (RDP) databases were anomalous, with the vast majority of these anomalies being chimeric sequences. Several computer programmes are now available to identify chimeric sequences within a dataset, e.g. the Pintail and Mallard programmes (Ashelford et al., 2005, 2006) and Bellerophon (Huber et al., 2004). Chimeric sequences can affect any target amplified by PCR, not only 16S rRNA templates.

Aside from the possibility of generating spurious 16S rRNA gene sequences during PCR, another potential problem is the well documented occurrence of multiple rRNA operons within a single prokaryotic genome (Stewart et al., 1982; Johansen et al., 1996), which, whilst usually identical, may show differing sequences (Mylvaganam and Dennis, 1992). Such variable sequences confound estimates of population diversity based on rRNA sequences, which most likely over-estimate the diversity within a population (Crosby and Criddle, 2003). For instance, an individual organism containing multiple, variable, copies of the 16S rRNA gene within its genome, may appear as multiple bands on a DGGE gel (Nubel et al., 1996).

Perhaps most significantly, PCR based amplification of complex microbial populations are entirely dependent on the presence of appropriate complementary primer sequences for the target gene of interest. These primers will inevitably have been designed based on previously studied gene sequences, often from cultivated bacterial strains. As such, it is probable that a proportion of the overall diversity within a population is missed by PCR due to sequence heterogeneity.

1.8 The Importance of Cultivation Independent Analysis of Bacterial Populations

Halocarboxylic acid dehalogenases have been studied in great detail, principally by cultivation dependent methods, where organisms have been enriched from an environmental inoculum and studied as pure cultures in the laboratory. However, such studies are subject to cultivation biases and may not reflect dehalogenating systems in the natural environment. For instance, Dunbar et al. (1997) showed that the diversity of *tfdA* and *tfdB* genes (encoding enzymes catalysing the first steps of the 2,4-dichlorophenoxyacetic acid (2,4-D) degradation pathway) from isolates obtained following batch culture enrichment was much narrower than those obtained from direct plating of the original population. Similarly, Watanabe et al. (1998) showed that the dominant phenol hydroxylase genes from activated sludge were lost during batch culture enrichment. Marchesi and Weightman (2003b) showed, using combined cultivation dependent and independent methods, that dehalogenases present after batch culture enrichment were more closely related to dehalogenases previously identified by cultivation methods, than to the original dehalogenase gene pool in activated sludge from which they originated.

These studies highlight the importance of cultivation-independent study of microbial populations to gain greater insight into the natural environmental degradation of xenobiotics. Therefore, in order to gain a comprehensive view of catabolic microbial populations using cultivation-independent methods, it is necessary to carry out simultaneous identification of both catabolic and 16S rRNA genes (Watanabe and Baker, 2000).

1.9 Project Aims

The principle aim of this project was to employ a cultivation-independent approach to investigate RBT outcomes beyond a simple pass or fail results, according to the bacterial community changes taking place during acclimation and degradation of αHAs. The αHAs were chosen because they include both very degradable and more recalcitrant compounds (Ellis et al., 2001), and molecular genetic methods are available with which to assess the catabolic capability of the microbial population enriched by these substrates (Hill et al., 1999). From previous studies, it was anticipated that substrate degradation in RBTs, measured by dechlorination, would be

linked to shifts in the bacterial community structure and the presence and expression of αHA dehalogenase genes.

Due to the inherently variable composition of activated sludge, Chapter 3 describes an investigation into the preparation of a standardized inoculum for use in RBT model tests. In order to maintain as much diversity as possible within the inoculum, this was attempted by directly lyophilising activated sludge without any additional pre-treatment. It was hypothesised that the activity of lyophilised activated sludge with αHAs dichloroacetic acid (DCA) and 2-monochloropropionic acid (2MCPA) would be comparable to that of fresh sludge, as measured by RBT outcome. It was also hypothesised that molecular genetic analysis of the bacterial community during RBTs would link community composition and functionality (RBT pass/fail).

Although the effect of inoculum source on the outcome of RBTs has been extensively studied, little attention has been paid to inoculum size. Therefore, Chapters 4 and 5 describe an experimental programme designed to investigate the effects of inoculum size on the outcome of RBTs using 2MPCA (Chapter 4) and trichloroacetic acid (TCA) (Chapter 5), as examples of relatively easily degraded and more recalcitrant compounds, respectively. It was hypothesised that higher inoculum concentrations would carry a greater degradative capacity for both chemicals, resulting in RBT pass results, whilst lower concentrations would have reduced degradative capacity resulting in RBT fails. It was also hypothesised that molecular genetic analysis would provide a link between community activity/inactivity and RBT pass/fail.

Two bacteria, able to grow on αHAs as a sole sources of carbon and energy, were isolated from these studies, and were characterised in terms of their identification and their dehalogenases, as described in Chapter 6.

2. Materials and Methods

2.1 Bacterial Strains and Growth Conditions

Bacterial strains and plasmids used in this study are shown in Table 2.1. Unless indicated otherwise, *Pseudomonas putida* strain PP3, *Pseudomonas* sp. strain ML1 and *Rhodococcus* sp. strain R1 were grown in Standard Basal Salts (SBS) minimal medium (Slater et al., 1979) containing 5 mM halogenated substrate, with or without 5 mM acetate, at 30°C with shaking at 150 rpm. All halogenated substrates were prepared as 1 M stocks, adjusted to pH 6.8 using NaOH and filter sterilised with 0.2 μm filters (Sartorius, UK). SBS was also prepared as solid media by addition of 1.5% (w/v) agar.

Escherichia coli was grown in LB medium (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl – MP Biomedicals) or SOC medium (Invitrogen) at 37°C on solidified media (1.5% agar), or in liquid with shaking at 150 rpm. All growth media prepared in this study was sterilised by autoclaving at 121°C for 15 min (15 psi).

Table 2.1 Bacterial strains and vectors used in this study

Strain / vector	Source
Pseudomonas putida strain PP3	(Senior et al., 1976; Weightman et al., 1979)
Pseudomonas sp. strain ML1	This study
Rhodococcus sp. strain R1	This study
Escherichia coli JM109	Promega
pGEM®-T Easy vector	Promega

2.2 Modified Ready Biodegradation Testing and Sampling

The organisation for economic co-operation and development (OECD) provide guidelines for testing the biodegradability of chemicals (see chapter 1 for details), including several ready biodegradation tests (RBTs). The following test is a modified version of the OECDs 301 test protocols (OECD, 1992).

Filter sterilised αHA (20 mg carbon/l) were incubated in diffuse light at 22°C in 1 L conical flasks containing 500 ml sterile mineral media (OECD, 1992) with an additional 1 mM NaCl, using an activated sludge (30 mg/l dry suspended solids, unless indicated otherwise) or 0.8% (w/v) soil inoculum and shaken at 170 rpm.

Enrichment cultures were maintained under these conditions until 100% dechlorination was achieved.

Samples were removed aseptically for Cl^- analysis (1.6 ml) and nucleic acid extraction (3.4 ml). Time zero samples were taken immediately after inoculation, followed by further samples every second day or according to the biodegradation kinetics of the substrate. Samples for nucleic acid extraction were stored at -20°C until ready for processing. Chloride measurements were taken immediately and performed in triplicate, as described in section 2.2.1.

2.2.1 Chloride assays

Chloride release was monitored for samples from RBT systems (section 2.2) as a measure of substrate dechlorination, and for samples taken from dehalogenase assays (section 2.4) as follows: 500 µl aliquots of unknown Cl^- concentration were added to a gradiented beaker (Sherwood Scientific Ltd.) containing; 13 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) and measured for Cl^- concentration using a Sherwood chloride analyser model 926 (Sherwood Scientific Ltd.) according to the manufacturer's instructions.

2.2.2 Inoculum sampling, preparation and storage

Activated sludge was collected from the aerobic basin of Coslech waste water treatment plant (WWTP) – situated to the West of Cardiff (51°30'7.00"N, 3°20'37.16"W) – which treats municipal waste water with some industrial waste water input. Sludge was sampled on 31 January 2007 (Chapter 3); 30 June 2008 (Chapter 4); 18 February 2009 (Chapter 5). Freshly collected activated sludge was kept aerated on returning to the laboratory until dry weights were determined, or lyophilised immediately for storage. Dry weights were determined by filtering 5 ml sludge aliquots through dried, pre-weighed glass microfiber filters (Whatman Ltd) and drying for 3 h at 180°C. Filters were re-weighed and the dry weight calculated. For storage by lyophilisation, fresh sludge was centrifuged at 20,000 ×g for 20 min at 20°C. The supernatant was removed and discarded, leaving 5-10 ml liquor with which to re-suspend the pelleted sample. Re-suspended sludge pellets were dispensed as 1 ml

aliquots, snap frozen in an acetone - dry ice bath and lyophilised under vacuum. Samples were then stored at -20°C for future use.

Soil was collected from a coastal lowland rainforest at Daintry, near Cape Tribulation, North Queensland (16°6'11.00"S, 145°26'49.00"E) and cooled in an ice box for transport back to the UK. Soil was then used directly for RBT enrichments as described above.

2.3 Preparation of Cell Free Extracts (CFE)

Cultures grown for the preparation of a cell free extract (CFE) were grown in SBS containing 13.8 mM 2MCPA, 20.8 mM acetate and 0.01% (w/v) yeast extract at 30°C with shaking at 150 rpm until 80-90% dechlorination was achieved (mid-late log growth phase). Cells were harvested by centrifugation at 9,800 ×g for 10 min at 4°C, washed twice in ice cold 50 mM Tris-H_2SO_4 buffer (pH 7), and finally re-suspended in 10-15 ml of the same buffer, with addition of 2 mM dithiothreitol (DTT). Cells were lysed by passage through a pre cooled (4°C) French pressure cell twice at 1260 psi. Cellular debris was removed from the lysate by centrifugation at 48,400 ×g for 30 min at 4°C. The clear CFE was removed and kept on ice until ready for dehalogenase activity assay, or aliquoted and stored at -80°C for future use. The pelleted cell debris was re-suspended in 5 ml 50 mM Tris-H_2SO_4 buffer (pH 7) with DTT to a final concentration of 2 mM and kept on ice for immediate dehalogenase activity assay, or stored at -80°C for future use.

2.4 Dehalogenase Assay

Dehalogenase assays were performed in glass test tubes in a total volume of 5 ml according to the method of Thomas et al. (1992a). 250 µl CFE was added to 200 mM Tris-H_2SO_4 buffer (pH 8) containing 1 mM NaCl and equilibrated at 30°C for 5 min. The assay was started on the addition of substrate to a final concentration of 50 mM. Aliquots of 500 µl were removed at regular intervals over a 30-40 min period and assayed for Cl^- release as described in section 2.2.1. CFE were routinely assayed for activity with MCA, DCA and 2MCPA. The protein concentration in the CFE was estimated using the Bradford reagent (Sigma), according to the manufacturer's instructions, using bovine serum albumin (Sigma) for calibration of the assay. Absorbance was measured at 595 nm where the amount of absorption is proportional

to the protein concentration (Bradford, 1976). One unit of dehalogenase activity was defined as the amount of enzyme required to convert 1 μmol substrate / min.

2.5 Nucleic Acid Extraction

Samples from RBT flasks (2 ml) or from pure cultures (1 ml) were concentrated by centrifugation and used for genomic DNA extraction using MP Biomedicals FastDNA®SPIN Kit for soil according to the manufacturer's instructions. Briefly, samples were lysed by bead beating using lysing matrix E; cell debris and lysing matrix were removed by centrifugation, and DNA was purified from the supernatant using silica based spin filters. This method has been shown previously to be a rapid and efficient way of extracting high quality DNA from environmental samples (Webster et al., 2003).

Total cellular RNA was extracted from 1 ml samples taken from biodegradation tests using QIAGENs RNeasy® Mini Kit following the manufacturer's instructions, with a modified cell lysis procedure. Cells were harvested by centrifugation at 16,100 ×g for 3 min and subjected to a lysozyme digestion (2 mg/ml lysozyme in TE buffer; 10 mM Tris, 1 mM EDTA, pH 8) for 10 min, followed by sonication on ice for 5 × 10 s bursts at 5 Hz using a Soniprep 150 (Wolf Laboratories Ltd). Lysates were treated according to the manufacturer's instructions. Following RNA extraction, samples were treated with RQ1 RNase-free DNase (Promega) to remove genomic DNA and stored at -80°C until required.

2.6 Reverse Transcriptase (RT) and PCR Amplification

All PCR and RT reactions were carried out using a DNA engine Dyad™ thermal cycler gradient block (MJ Research). PCR reactions were performed using Promega's GoTaq® Flexi DNA Polymerase and deoxynucleotide triphosphate reagents and made up to the indicated reaction volume using diethylpyrocarbonate (DEPC) treated RNase free water (Severn Biotech). All laboratory equipment used during (RT)-PCR preparation was treated with a UV cross linker (UVP) prior to use. Primer sequences used throughout this study are listed in Table 2.2.

Table 2.2 Oligonucleotide primers used in this study for PCR and cDNA synthesis

Primer name	Target gene	Primer sequence (5' - 3')[a]	Reference
357F[b, c]	16S rRNA gene	CCTACGGGAGGCAGCAG	(Muyzer et al., 1993)
518R[d]	16S rRNA gene	ATTACCGCGGCTGCTGG	(Muyzer et al., 1993)
27F	16S rRNA gene	AGAGTTTGATCMTGGCTCAG	(Lane, 1991)
907R	16S rRNA gene	CCGTCAATTCMTTTGAGTTT	(Lane, 1991)
1492R	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)
M13F		GTAAAACGACGGCCAGT	
M13R		CAGGAAACAGCTATGAC	
$dehI_{for1}$	*dehI*	ACGYTNSGSGTGCCNTGGGT	(Hill et al., 1999)
$dehI_{rev1}$	*dehI*	AWCARRTAYTTYGGATTRCCRTA	(Hill et al., 1999)
$dehI_{rev2}$	*dehI*	SGCMAKSRCNYKGWARTCACT	(Hill et al., 1999)
$dehII_{for1}$	*dehII*	TGGCGVCARMRDCARCTBGARTA	(Hill et al., 1999)
$dehII_{rev1}$	*dehII*	TCSMADSBRTTBGASGANACRAA	(Hill et al., 1999)
OMPf	*coxL*	GGCGGCTTYGGSAASAAGGT	(King, 2003)
BMSf	*coxL*	GGCGGCTTYGGSTCSAAGAT	(King, 2003)
O/Br	*coxL*	YTCGAYGATCATCGGRTTGA	(King, 2003)
270		TGCGCGCGGG	(Mahenthiralingam et al., 1996)
272		AGCGGGCCAA	(Mahenthiralingam et al., 1996)
277		AGGAAGGTGC	(Mahenthiralingam et al., 1996)

a. IUPAC ambiguity code used: 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. For DGGE, this primer had an additional 5' GC clamp: CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG (Muyzer et al., 1993) and is referred to as 357FGC in this report.

c. For re-amplification of excised DGGE bands, this primer had a 5' GC linker, which also contains an M13R site: CAGGAAACAGCTATGACGGGCGGGGCGGGGGCACGGGGGG (O'Sullivan et al., 2008) and is referred to as 357FM13R in this report.

d. 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 518RM13F in this report.

2.6.1 16S rRNA gene PCR

The 16S rRNA gene positions 357-518, 27-907 and 27-1492 (based on *E. coli*; Brosius et al., 1981), were amplified using the primers pairs 357FGC and 518R (Muyzer et al., 1993), 27F and 907R or 27F and 1492R (Lane, 1991). The PCR mixture was made up in a 50 µl volume consisting of 1 × buffer; 1.5 mM $MgCl_2$; 0.25 mM dNTPs; 0.2 µM of both 357FGC and 518R or 27F and 907R or 27F and 1492R; 1.25 U *Taq* DNA polymerase and 1 µl template DNA. The following conditions were used for PCR amplification using 357FGC and 518R: 95°C for 5 min; 10 × cycles of 94°C for 30 s, 55°C for 10 s, 72°C for 1 min; 25 × cycles of 92°C for 30 s, 52°C for 30 s, 72°C for 1 min; 72°C 10 min. Conditions for PCR amplification using 27F and 907R/1492R were: 95°C for 2 min; 36 × cycles of 94°C for 30 s, 52°C for 30 s, 72°C for 1.5 min; 72°C 5 min.

2.6.2 DNA gyrase subunit B (*gyrB*) gene PCR

Subunit B of DNA gyrase was amplified using the primers UP-1 and UP-2r (Yamamoto and Harayama, 1995). The PCR mixture (50 µl) contained 1 × buffer; 1.5 mM $MgCl_2$; 0.2 mM dNTPs; 1 µM of UP-1 and UP-2r; 2.5 U *Taq* DNA polymerase and 1 µl template DNA. The following conditions were used for PCR amplification: 94°C for 5 min; 30 × cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 2 min; 72°C 5 min. Two specifically designed primers, UP-1S and UP-2Sr, were used for sequencing the *gyrB* PCR products.

2.6.3 α-HA dehalogenase gene PCR

The degenerate PCR primers described by Hill et al (1999) were used to amplify the α-HA dehalogenases *dehI* (*dehI* short ca. 230 bp, $dehI_{for1}$ & $dehI_{rev1}$; *dehI* long ca. 504 bp, $dehI_{for1}$ & $dehI_{rev2}$) and *dehII* (ca. 422 bp). PCR amplification of dehalogenase genes was performed in a 50 µl reaction containing 1× buffer; 1.5 mM $MgCl_2$; 0.2 mM dNTPs; 4 µM $dehI_{for1}$ and $dehI_{rev1}$/$dehI_{rev2}$ or 6 µM $dehII_{for1}$ and $dehII_{rev1}$ primers; 1 U *Taq* DNA polymerase, and 1 µl template DNA. The following touchdown PCR cycle was used for *dehI*: 94°C for 2 min; 20 × cycles of 92°C for 20 s, 70°C for 30 s (-1°C per cycle), and 75°C for 30 s; 20 × cycles of 92°C for 20 s, 51°C for 30 s, and 75°C for 30 s; 75°C for 5 min. The PCR cycle for *dehII* was: 94°C for 10 min; 36 × cycles of 94°C for 45 s, 55°C for 2 min, 75°C for 45 s; 75°C for 5 min.

2.6.4 Carbon monoxide dehydrogenase large subunit (*coxL*) gene PCR

Form I and form II *coxL* genes were amplified using the primers OMPf, BMSf and O/Br described by King (2003) in a 50 µl mix containing 1× buffer; 3.5 mM $MgCl_2$; 0.1 mM dNTPs; 0.1 µM OMPf / BMSf and O/Br; 1.25 U *Taq* DNA polymerase and 1 µl template DNA. The following touchdown PCR cycle was used: 94°C for 5 min; 8 × cycles of 94°C for 45 s, 62°C for 1 min (-1°C every second cycle), 72°C for 1.5 min; 30 × cycles of 94°C for 45 s, 58°C for 1 min, 72°C for 1.5 min; 72°C for 5 min.

2.6.5 PCR amplification of cloned inserts

PCR amplification of cloned inserts was performed using the reaction mixture described for 16S rRNA gene above with the M13F and M13R primers (0.2 µM each), using the following cycle: 94°C for 10 min; 30 × cycles of 92°C for 45 s, 50°C for 45 s, 75°C for 1 min; 75°C for 5 min.

2.6.6 First strand (cDNA) synthesis (RT-PCR)

RT and PCR reactions were performed as separate reactions. First strand (cDNA) synthesis was carried out using Invitrogen's SuperScript™ III reverse transcriptase according to the manufacturer's instructions, as follows (concentrations given are for the final 20 µl reaction volume): 2-5 µl extracted RNA, 0.5 mM dNTPs, 0.1 µM of 518R, or 2 µM $dehI_{rev1}$ / $dehI_{rev2}$, or 3 µM $dehII_{rev1}$ was made up to 14 µl using DEPC treated RNase free water (Severn Biotech). This mixture was heated at 65°C for 5 min, then placed immediately on ice for 2 min, before adding First strand buffer to × 1 concentration, 5mM DTT and 200 U SuperScript™ III RT (making a final volume of 20 µl). This reaction was then incubated at 55°C for 45 min, and the 70°C for 15 min. Each RT reaction was routinely accompanied by two separate control reactions containing the mixture above, with a) no template RNA, and b) no RT enzyme, to detect any RNA contamination of the reagents or genomic DNA contamination of the extracted RNA. The resulting cDNA was used for subsequent PCR amplification using appropriate primer combinations as described above, or stored at -80°C until required.

2.6.7 Random amplified polymorphic DNA (RAPD) analysis

The RAPD primers (10-mers) 270, 272 and 277 (Mahenthiralingam et al., 1996) were used in a 50 µl mixture containing 1× buffer; 3 mM $MgCl_2$; 0.25 mM dNTPs; 1.6 µM

270 or 272 or 277 primer; 1.25 U *Taq* DNA polymerase and 2 µl template DNA. The following PCR cycle was used: 4 × cycles of 94°C for 5 min, 36°C for 5 min, 72°C for 5 min; 30 × cycles of 94°C for 1 min, 36°C for 1 min, 72°C for 2 min; 72°C for 10 min.

2.7 ELECTROPHORESIS METHODS

2.7.1 Agarose gel electrophoresis

All PCR products and genomic DNA were routinely checked and quantified using 1.2% (w/v) agarose gels prepared in 1 × TAE buffer (pH 8.3; 40 mM Tris-acetate, 1 mM EDTA – Severn Biotech) stained with SYBR safe™ DNA gel stain (Invitrogen) and Hyperladder I (Bioline) and run at 120 V for 25 min. Gels were photographed using the Gene Genius Bio Imaging System (Syngene). RAPD PCR products were run on 1.5% (w/v) agarose gels prepared in 1 × TAE buffer and run at 100 V for approximately 40 min, before staining with ethidium bromide (0.5 µg/ml). Gels were photographed as above.

2.7.2 Denaturing gradient gel electrophoresis (DGGE)

DGGE analysis was performed using the DCode™ universal mutation detection system (Biorad laboratories) according to the method of Muyzer et al. (1993), with modifications as described by Webster et al. (2006). PCR products (ca. 150 ng) amplified using primers 357FGC and 518R were loaded onto a polyacrylamide gel (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 a 50 ml volume Gradient Mixer (Fisher Scientific, UK). Electrophoresis was carried out in 1 × TAE buffer at 80 V for 10 min, and then at 200 V for 4.8 h at 60°C. Polyacrylamide gels were stained using SYBR® gold nucleic acid gel stain (Invitrogen) for 20 min and visualised under UV illumination. Gel images were captured using the Gene Genius Bio Imaging System (Syngene).

DGGE bands were excised from polyacrylamide gels using a scalpel and washed in DEPC treated water for 10 min, then air dried for 10 min. Dried bands were crushed and re-suspended in 15 µl DEPC treated water and cooled at -20°C over night before use, or stored at -20°C until required. This suspension (1 µl) was used as template

DNA for subsequent re-amplification using primers 357FM13R & 518RM13F (Table 2.2; O'Sullivan et al., 2008) and the reaction conditions described for the 16S rRNA gene, positions 357-518 above. Re-amplified PCR products were sequenced using either M13F or M13R.

2.7.3 Native polyacrylamide gel electrophoresis (PAGE)

PAGE was conducted according to the method of Thomas et al. (1992a) with modifications. Electrophoresis was performed using the DCode™ universal mutation detection system (Biorad laboratories). The running gel consisted of 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 DTT, 0.1% ammonium persulphate and 0.075% tetramethylethylenediamine (TEMED). The stacking gel consisted of 123 mM Tris-H_2SO_4 buffer (pH 6.8), 3% acrylamide (consisting of an acrylamide:bis-acrylamide ratio of 37.5:1), 0.1% ammonium persulphate and 0.05% TEMED. The running buffer (pH 8.3) consisted of 192 mM glycine, 25 mM Tris and 1 mM DTT. CFE (50-200 µl) was loaded onto gels with bromophenol blue to track electrophoresis, and run at 200 V for 6 – 9 h at 4°C. Following electrophoresis, gels were incubated in 0.2 M Tris-H_2SO_4 buffer (pH 7.9) containing 50 mM halogenated substrate, at 30°C, for 15-30 min. Gels were then washed twice in deionised H_2O and immersed in 100 mM $AgNO_3$ until silver chloride precipitation was observed (1-2 min), corresponding to the position of a dehalogenase. Gels were then washed in deionised H_2O and fixed in 5% acetic acid.

2.8 Dehalogenase Gene Cloning

Clone libraries were constructed from PCR products amplified from either genomic DNA (PCR) or total RNA (via RT and PCR) templates. Each library was constructed from 3 independent, pooled PCR reactions and cleaned using Freeze 'N Squeeze DNA Gel Extraction Spin Columns (Biorad laboratories) according to the manufacturer's instructions, where PCR products were extracted from an agarose plug by freezing, and subsequently centrifuging to collect the purified PCR product (Thuring et al., 1975). Samples were then concentrated using Montage PCR Centrifugal Filter Devices (Millipore), according to the manufacturer's instructions. Cloning was done using the pGEM-T easy vector system (Promega, Figure 2.1) and *Escherichia coli* JM109 (Yanisch-Perron et al., 1985) competent cells (Promega) according to the

manufacturer's instructions with modifications. Briefly, ligation reactions contained 1 × ligation buffer, 50 ng vector, 3 U T4 DNA ligase and PCR product (1:1 molar ratio of template to plasmid), made up to 10 µl using DEPC treated RNase free water (Severn Biotech). Ligations were incubated overnight at 4°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 47 s at 42°C, then returning immediately to ice for a further 2 min. Cells were revived in 950 µl SOC medium (Invitrogen) 37°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°C for 24 h for selection of transformants.

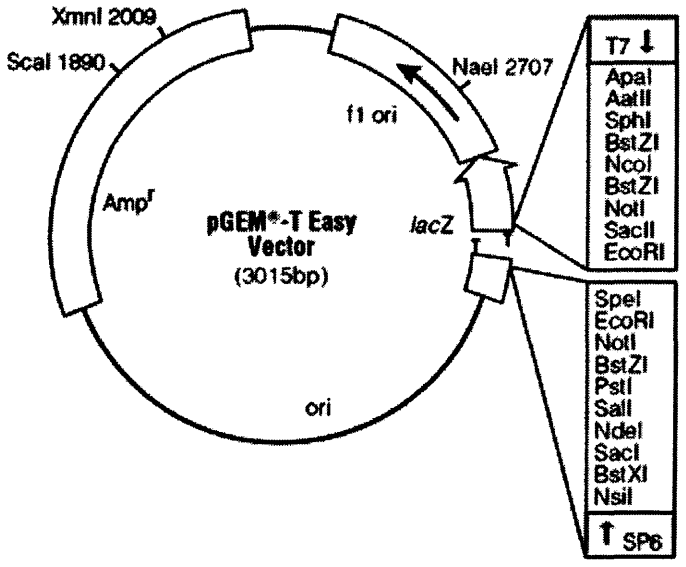

Figure 2.1 pGEM®-T Easy vector. This high copy number linearlised vector contains 3' T overhangs within the multiple cloning site (MCS) allowing the simple and efficient ligation of PCR products due to the presence of the compatable A overhang produced by Taq polymerase. Ligation into the *lacZα* gene allows for blue white screening.

Blue white selection is achieved by a process termed alpha complementation (Moosmann and Rusconi, 1996). The *lacZα* 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 lacZα gene is disrupted following the ligation of a nucleic acid fragment into the multiple cloning site (MCS), alpha complementation of *LacZΩ* can not be achieved, and therefore no beta-galactosidase is formed, thus colonies will be white.

Significant numbers of blue colonies were found to contain cloned inserts, most likely due to the formation of a hybrid insert:LacZα protein which maintained its functionality, which was likely due to the short length of the inserts amplified in this study. Therefore both blue and white colonies were picked in equal numbers. Transformants were picked into a 96 well plate (Thermo Scientific Nunc) containing LB broth with ampicillin (100 µg/ml) and 10% (v/v) glycerol (for cryoprotection of stored clones), leaving 2 wells without clones as controls, and incubated over night at 37°C with shaking at 150 rpm. Each transformant containing well was screened for cloned inserts by PCR (see section 2.6.5) and agarose gel electrophoresis. Insert containing clones were picked at random from each library and sequenced using the M13F or M13R primer. 96 well plates containing transformants were stored at -80°C for future use.

2.9 Data analysis

2.9.1 DGGE gel analysis

The DGGE band positions and intensities were determined using GelCompar II software (Applied Maths, Belgium). Bands were matched with 1% position tolerance and 1% optimisation. A similarity matrix was calculated based on the Pearson coefficient and used to construct a dendrogram using the unweighted pair-group method with arithmetic averages (UPGMA).

2.9.2 DNA sequencing and phylogenetic analysis

Sequencing reactions were performed using the ABI PRISM BigDye Terminator v3.1 cycle sequencing kit and run on an ABI 3130×1 Genetic Analyzer. Sequences were viewed using Chromas lite v2.01 (Technelysium Pty Ltd) and their closest matches identified using the basic local alignment search tool (BLAST) and The Ribosomal Database Project (RDP). Sequences were aligned with references from GenBank using ClustalW2 (Larkin et al., 2007), then checked and manually edited using

BioEdit (Hall, 1999c). Primer regions were removed from all dehalogenase sequences prior to alignment to avoid the generation of false variability caused by the degenerate nature of the primers. Phylogenetic trees were routinely constructed using both nucleotide and derived amino acid sequences in Mega v4.0 (Tamura et al., 2007) by the Neighbour-Joining method (Saitou and Nei, 1987) using the Jukes and Cantor correction algorithm (Jukes and Cantor, 1969), although other methods (including the Neighbour Joining and Minimum Evolution methods using P-distance or log-det algorithms) were also used to validate the derived tree topology. However, all trees displayed in this study are based on nucleotide sequence alignments. The calculated bootstrap values from 1000 replicates are shown as percentages at the nodes.

The partial nucleotide sequences of the DNA gyrase subunit B gene, dehalogenase genes and 16S rRNA genes amplified in this study have been deposited in the EMBL database under the following accession numbers:

Chapter 3:	partial dehalogenase genes – FN997661 to FN997679
	partial 16S rRNA genes – FR657516 to FR657518
Chapter 4:	partial dehalogenase genes – FN998842 to FN998870
	partial 16S rRNA genes – FR657519 to FR657527
Chapter 5:	partial dehalogenase genes – FR647445 to FR647484
	partial 16S rRNA genes – FR657528 to FR657531
Chapter 6:	partial 16S rRNA genes – FN825677 and FN825678
	partial dehalogenase genes – FR666698 and FR666699
	partial *gyrB* gene – FR666697

3. Inoculum Standardisation and Analysis of Bacterial Community and Catabolic Gene Changes During Ready Biodegradation Tests (RBTs) Using α-Halocarboxylic Acids as Test Substrates

3.1 Introduction

The microbial diversity of activated sludge has been the subject of numerous studies. Generally, sludges are dominated by members of the *Alpha*, *Beta* and *Gamma Proteobacteria*, as well as the *Bacteroidetes* and *Actinobacteria* (Wagner and Loy, 2002). Despite this general trend, Forney et al. (2001) demonstrate that microbial communities in waste water treatment plants (WWTP) show tremendous variability, not only between different plants, but also temporal variability within a single plant, concluding that the characterisation of a "typical activated sludge" was simply impossible. The microbial composition of any activated sludge is influenced by WWTP operating procedures, the composition of the plants influent water, as well as on environmental factors such as the weather and climate (Tabka et al., 1993; Forney et al., 2001). Complex microbial communities, such as activated sludge, are routinely used as inocula for standard ready biodegradation tests (RBTs), and are often blamed for the apparently stochastic results associated with RBT testing (Thouand et al., 1995; Forney et al., 2001; Paixão et al., 2006; Vazquez-Rodriguez et al., 2007).

Due to the inherent lack of reproducibility associated with RBTs conducted using activated sludge inocula, several methods of inoculum standardisation are allowed by Organisation for Economic Co-operation and Development (OECD) protocols. These include centrifugation, decantation, filtration, or aerating sludges for 5-7 days under test conditions, whilst the modified MITI (I) protocol (301 C) requires the incubation of inoculum in a glucose-peptone containing medium prior to use (OECD, 1992). However, these methods are known to introduce their own biases. Vazquez-Rodriguez et al. (2007) investigated the effects of using a preconditioning period, where different activated sludges were incubated for 5-7 days under test conditions, on RBT outcome. They showed that despite successfully homogenising the activity of three sludges, based on their hydrolytic enzyme activity profiles in the API-ZYM test, the ability of two of the sludges to degrade the xenobiotic compound aniline, was lost. Such loss of functionality gives false negative RBT results. Forney et al. (2001)

describe a significant reduction of microbial diversity and a corresponding reduction in physiological potential of activated sludge, following the pre-treatment required in the MITI (I) RBT, outlined above. The use of specifically designed microbial consortia, prepared from combinations of bacteria commonly found in sewage or soil, have also been investigated as substitutes for activated sludge. These have shown similar biodegradative activities using several simple test chemicals, though their catabolic potential for xenobiotic degradation requires further investigation (Paixão et al., 2006). Previous studies have also investigated the use of commercially available microbial mixtures such as BI-CHEM1 BOD Seed (Novozymes Biologicals Inc., Salem, VA, USA) and BIOLEN M112 (Gamlen Industries S.A., St. Marcel-Vernon, France), as inocula for RBTs; however, such mixtures often differ in their activity compared to native environmental populations such as activated sludge (Paixão et al., 2003; 2006).

The test system used in the present investigation is a model of commonly used OECD ready biodegradability tests, using two α-halocarboxylic acids (αHAs), dichloroacetic acid (DCA) and 2-monochloropropionic acid (2MCPA), as reference chemicals. The biodegradation of αHAs has been well characterised, and is initiated by the cleavage of the carbon-halogen bond by microbial dehalogenases (Slater et al., 1997). The αHA dehalogenases have also been well characterised and are divided into two evolutionarily unrelated families; group I (*dehI*) and group II (*dehII*) dehalogenases (Hill et al., 1999), each with their own separate mechanism of hydrolytic dehalogenation (Kurihara and Esaki, 2008). Previous investigations of αHA degradation have focused principally on organisms producing dehalogenases that have been isolated following a period of conventional enrichment culture on a given αHA substrate, which are subject to the well known cultivation bias (Wagner et al., 1993). Little attention has been given to the bacterial community adaptation taking place during the degradation of αHAs, or indeed other pollutants.

There were two principle aims of the work described in this chapter. The first was to prepare a stable microbial inoculum as an activated sludge substitute, with which to investigate the underlying causes of variability in RBT results. If the inoculum used for each test is constant, then any variability within the test system must be due to the stochastic nature of microbial enrichment, as all other parameters are under

experimental control. This was attempted by directly preserving samples of an environmental inoculum, activated sludge, by lyophilisation. The second aim was to assess the efficacy of cultivation-independent investigation of the bacterial community response (16S rRNA) and catabolic (dehalogenase) gene selection during a RBT, as a method of directly relating test outcome to inoculum composition.

3.2 RESULTS

3.2.1 Degradation kinetics of selected αHA using fresh or lyophilised activated sludge as an inoculum

Fresh or lyophilised activated sludge was used to inoculate RBTs containing DCA or 2MCPA as the sole source of carbon and energy. Figure 3.1 shows the degradation curves from all four tests. DCA was totally dechlorinated in both the fresh (DCA-F), and lyophilised (DCA-L), sludge tests after 9 days, following an initial 7 day lag (Figure 3.1). Dechlorination of 2MCPA using fresh sludge (2MCPA-F) was also complete after 9 days. 2MCPA dechlorination using lyophilised sludge (2MCPA-L) gave a two phase dechlorination curve, following a lag period of approximately 10 days. Phase 1 started at day 10 and plateaued briefly around day 30, with the onset of phase 2 around day 36 through to complete dechlorination by day 44 (Figure 3.1). Both the DCA-F and DCA-L tests, and the 2MCPA-F test achieved the 70% dechlorination pass mark, required by the RBT, well within the 28 day test period. The 2MCPA-L test failed to achieve 70% dechlorination within the 28 day test period.

3.2.2 DGGE profiling of the bacterial population during the DCA-L test

Denaturing gradient gel electrophoresis (DGGE) of 16S rRNA genes and transcripts was used to assess the diversity of the bacterial population present in the DCA-L test, and to assess the sensitivity of the two nucleic acid templates to changes in the bacterial community. Figure 3.2 (A and B) shows DGGE profiles (from RNA and DNA, respectively) from the DCA-L test. A single phylotype, represented by bands D1 and D2, not present in the starting population (day 0) was enriched by day 8 in conjunction with the beginning of DCA degradation (see Figure 3.1) and persisted through to day 9. Excision and sequencing showed that bands D1 and D2 were identical and affiliated to the *Betaproteobacteria*, being most closely related to a *Ralstonia* sp. (Table 3.1). UPGMA analysis of DGGE profiles band sharing (Figure 3.3) showed little similarity (19%) between the DNA and RNA profiles from the starting population (day 0). DNA and RNA profiles from day 6 were also distinct (19% similarity) from one another, though the DNA and RNA profiles became much more uniform during substrate degradation (days 8 and 9) with all samples having at least 70% similarity (Figure 3.3).

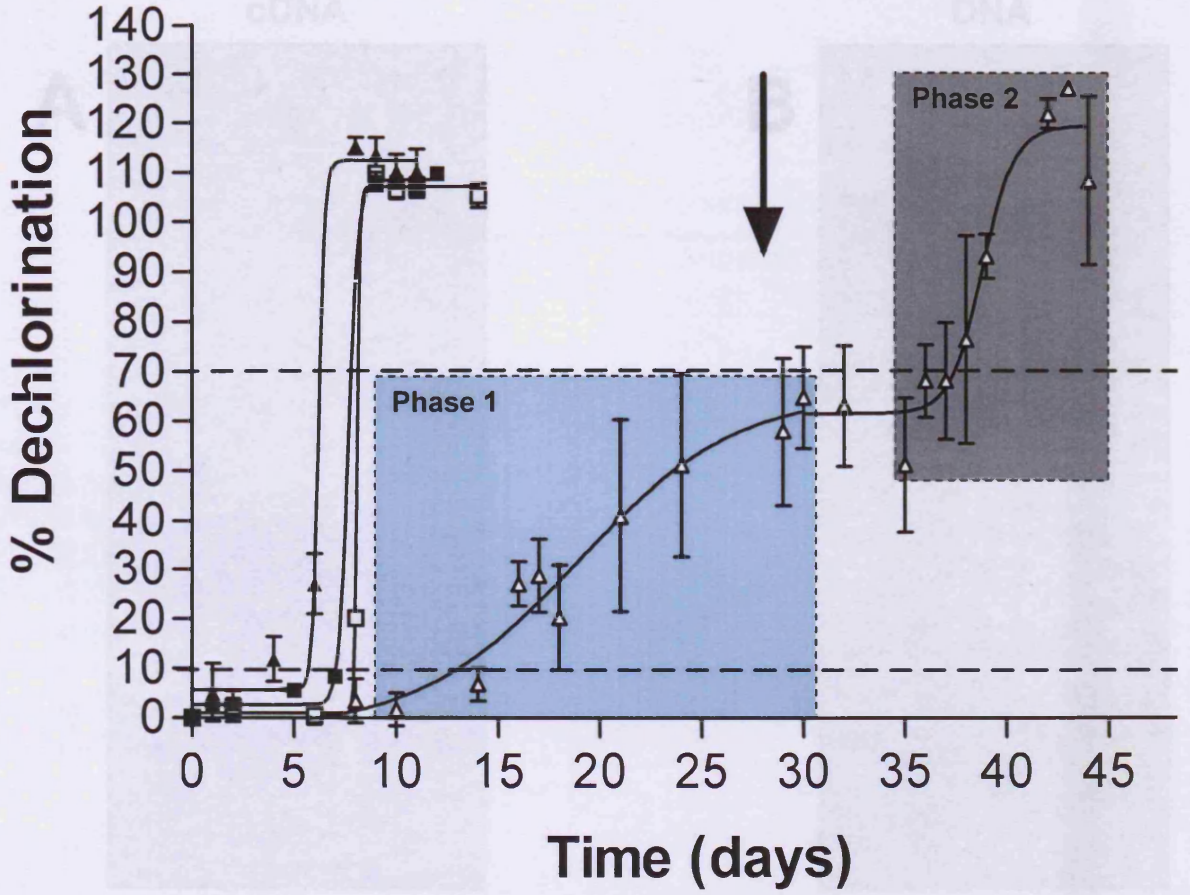

Figure 3.1 Degradation kinetics of αHAs inoculated with 30 mg/l fresh (closed symbols) or lyophilised (open symbols) activated sludge: DCA (■/□); 2MCPA (▲/△). Broken horizontal lines represent biodegradation start point (10%) and pass level (70%), and the arrow indicates the 28 day cut-off for a RBT pass according to OECD guidelines. Error bars represent the standard error of the mean (n = 3).

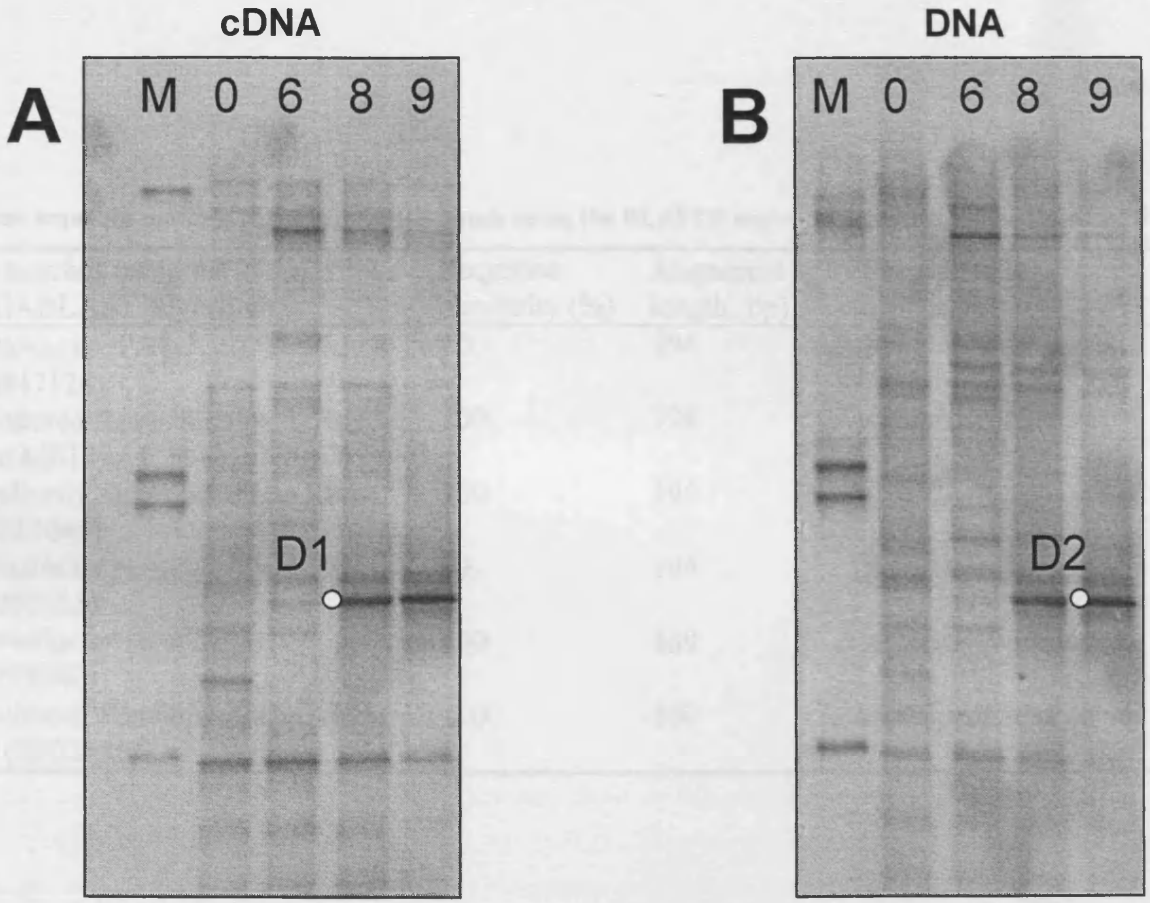

Figure 3.2 DGGE of the 16S rRNA gene and its transcript from the DCA-L test. (A) 16S rRNA profile from DCA-L test. (B) 16S rRNA gene profile from DCA-L test. Numbers above each lane represent time (days, see Figure 3.1) of sampling, M = marker. Open circles represent bands excised for sequencing.

Table 3.1 Closest 16S rRNA gene sequence matches to excised DGGE bands using the BLASTN search tool and Ribosome Database Project SEQMATCH tool.

DGGE band[a]	Substrate	Top matches using the MEGABLAST algorithm	Sequence similarity (%)	Alignment length (bp)	Phylogenetic affiliation	Top Matches using RDP
D1/D2	DCA	*Ralstonia* sp. PA1-3 (DQ847128)	100	194	*Betaproteobacteria*	Ralstonia sp. PA1-3 (DQ847128)
		Uncultured *Betaproteobacterium* clone MS129A1_H07 (EF704792)	100	194	*Betaproteobacteria*	
M1/M2	2MCPA	Uncultured bacterium clone S1-24 (EU015089)	100	194	-	*Methylibium petroleiphilum* PM1 (CP000555)
		Methylibium petroleiphilum PM1 (CP000555)	99	194	*Betaproteobacteria*	
M5/M6	2MCPA	*Xanthobacter* sp. ROi16 (EF219040)	100	169	*Alphaproteobacteria*	*Xanthobacter flavus* 301 (X94199)
		Uncultured *Xanthobacter* sp. clone E10 (EF221817)	100	169	*Alphaproteobacteria*	

a. See Figures 3.2 and 3.4.

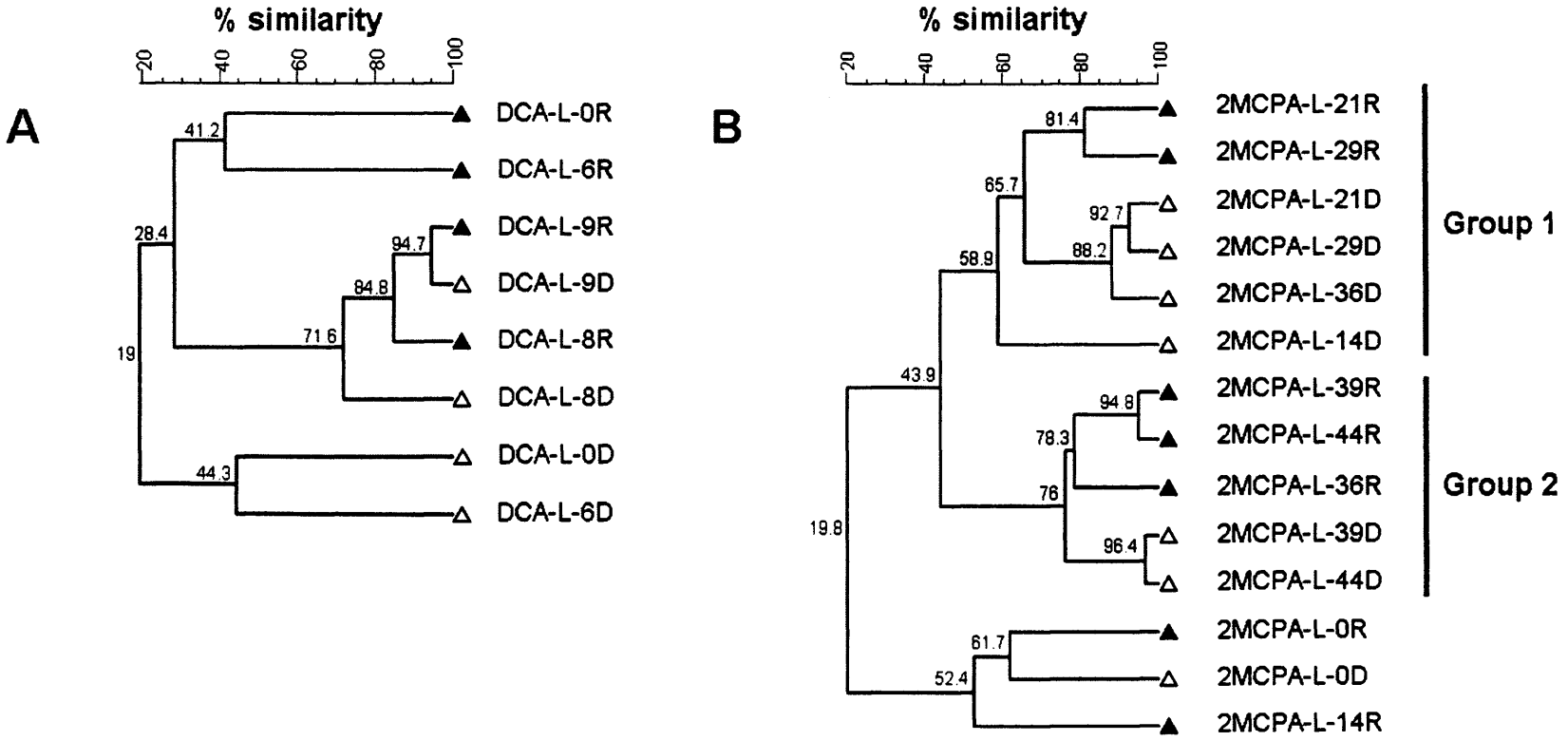

Figure 3.3 Dendrogram showing the % similarity between 16S rRNA gene and cDNA DGGE profiles from A) the DCA-L test, and B) the 2MCPA-L test. Open triangles represent samples amplified from DNA templates, closed triangles represent samples amplified from cDNA templates. Samples are identified as X-TN, where X = test ID (DCA-L or 2MCPA-L), T = sampling time (days), and N = nucleic acid template used for amplification (D = DNA, R = cDNA). The percentage similarity is indicated at each node. Cluster analysis was based on the Pearsons correlation using the unweighted pair-group method with arithmetic averages (UPGMA).

3.2.3 DGGE profiling of the bacterial population during the 2MCPA-L test

DGGE profiles (Figure 3.4) showed three different phylotypes were clearly enriched during 2MCPA dechlorination. Bands M1-M4 appeared during phase 1 at day 21 (see Figure 3.1) and gradually disappeared thereafter. Bands M5 and M6 emerged during phase 2 at day 36 (see Figure 3.1), and persisted through to day 44. None of these bands were detected in the starting population (day 0). Analysis of the partial 16S rRNA sequences from excised bands identified M1 and M2 as close relatives of the facultative methylotroph *Methylibium petroleiphilum* PM1 (Nakatsu et al., 2006), a *Betaproteobacterium*. Bands M5 and M6 were identical and most closely related to a *Xanthobacter* sp. (*Alphaproteobacteria*). No sequence could be obtained for bands M3 and M4. The closest relatives of all the dominant DGGE bands shown in Figure 3.4 are summarised in Table 3.1. UPGMA analysis clearly showed a distinction between the populations from phases 1 and 2 for both DNA and cDNA; with all samples from phase 1 clustering together in group 1 (Figure 3.3), and all samples from phase 2 clustering together in group 2, with the exception of sample 2MCPA-L-36D which was placed in group 1 (Figure 3.3). Similarity between DNA and cDNA profiles was generally high (> 65%), with the exception of samples from days 14 and 36, where the DNA and cDNA based profiles grouped separately.

3.2.4 Presence and expression of αHA dehalogenase genes in the DCA-L test

Samples from the DCA-L test were assayed for the presence of αHA dehalogenase genes and their transcripts using PCR primers described by Hill et al. (1999). Neither of the *dehI* primer sets gave PCR products at any stage during the DCA-L test (results not shown). However, *dehII* PCR products of the expected size (ca. 422 bp) were obtained from all time points analysed (Figure 3.5A), as well as *dehII* mRNA transcripts (Figure 3.5B), detected (as cDNA) at the beginning of DCA degradation (day 8) and towards the end of degradation (day 9).

3.2.5 Presence and expression of αHA dehalogenase genes in the 2MCPA-L test

Samples from the 2MCPA-L test were also assayed for αHA dehalogenase genes and their transcripts. *dehII* genes were present at all stages of the 2MCPA-L test (Figure 3.6A), but *dehII* mRNA was detected only transiently, with a peak at day 21

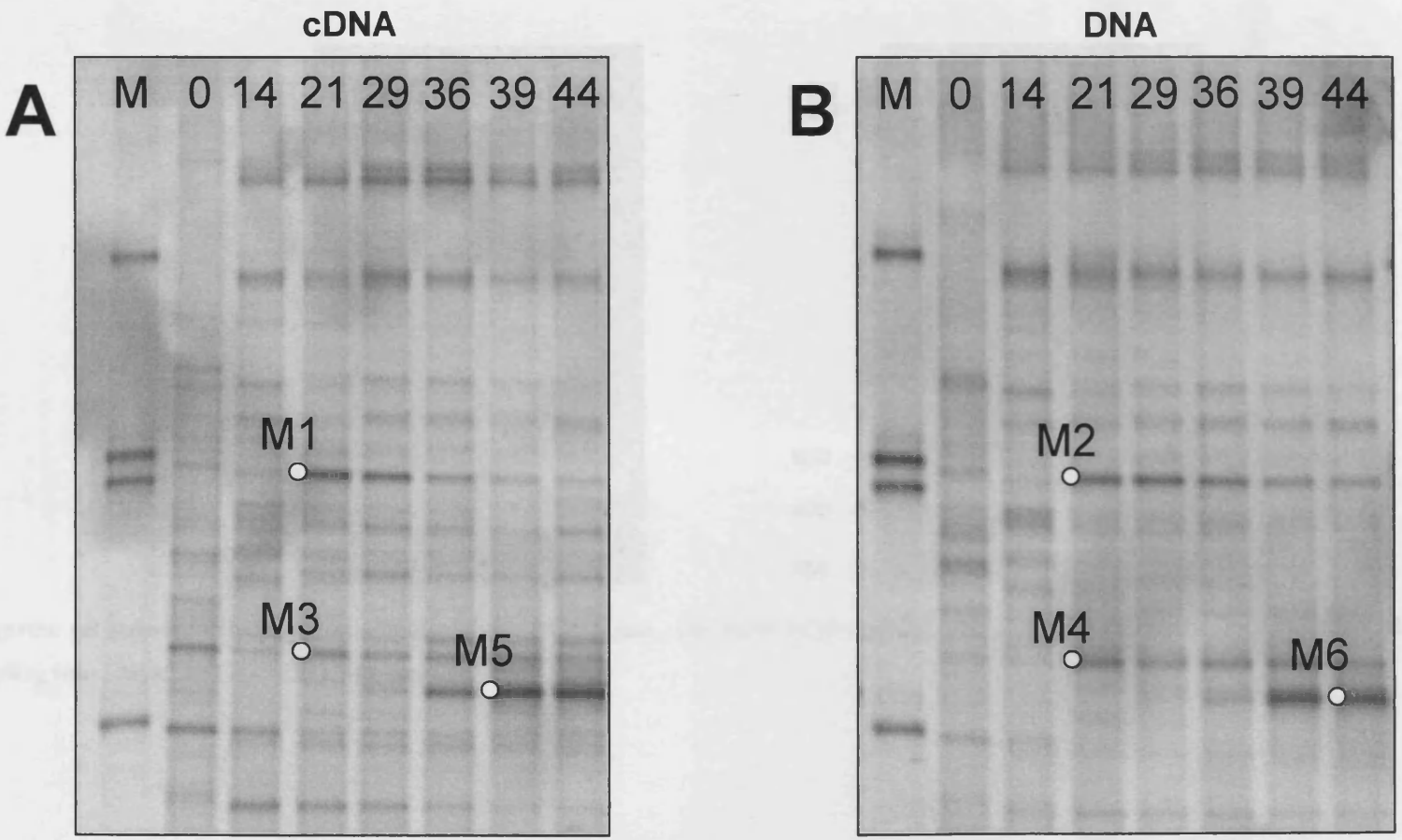

Figure 3.4 DGGE of the 16S rRNA gene and its transcript from the 2MCPA-L test. (A) 16S rRNA profile from 2MCPA-L test. (B) 16S rRNA gene profile from 2MCPA-L test. Numbers above each lane represent time (days, see Figure 3.1) of sampling, M = marker. Open circles represent bands excised for sequencing. Coloured circles represent phylogenetically unidentified bands.

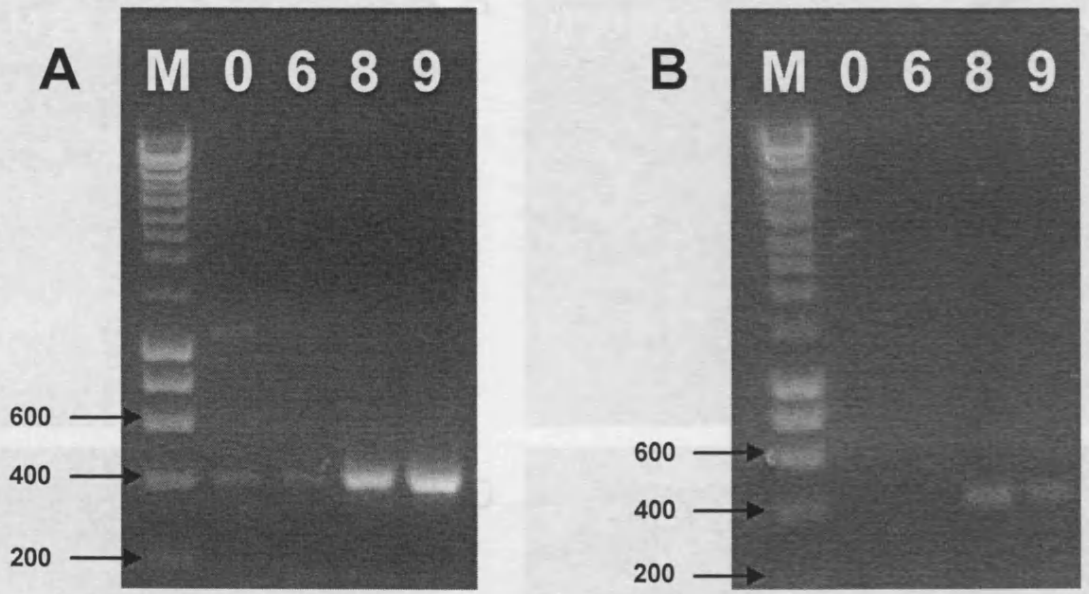

Figure 3.5 Agarose gel showing dehalogenases amplified from DCA-L test. (A) *dehII* PCR producs. (B) *dehII* RT-PCR products. Numbers above each lane represent sampling time (days; see Figure 3.1), M = marker.

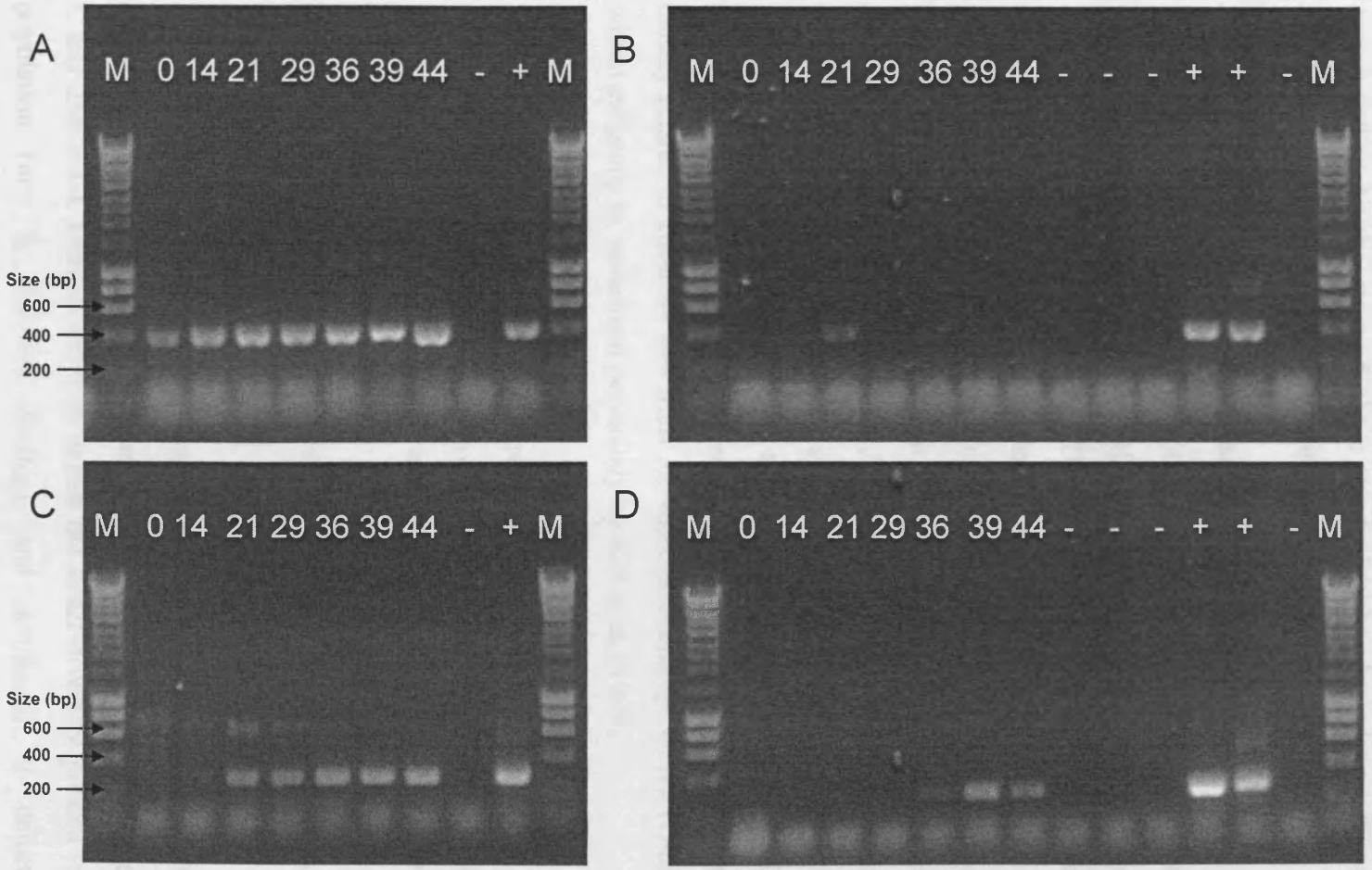

Figure 3.6 Agarose gel showing dehalogenases amplified from 2MCPA-L test. (A) *dehII* PCR producs. (B) *dehII* RT-PCR products. (C) *dehI* PCR products. (D) *dehI* RT-PCR products. Numbers above each lane represent sampling time (days; see Figure 3.1), + and – represent positive and negative controls respectively, M = marker.

(Figure 3.1; Figure 3.6B). Although *dehI* genes were not detected during the first 3 weeks of the test, a *dehI* product was observed from day 21 onwards (Figure 3.6C). As seen with *dehII*, *dehI* transcripts appeared transiently, first at day 36, corresponding to the start of phase 2 of 2MCPA degradation (Figure 3.1), and intensified by day 39, before a reduction by day 44 (Figure 3.6D).

3.2.6 Diversity of group I dehalogenase genes in the 2MCPA-L test

In order to assess the diversity of dehalogenase genes present during the 2MCPA-L test (*dehI* was not detected in the DCA-L test), clone libraries were prepared from *dehI* PCR products at day 39 (both DNA and mRNA templates; Figure 3.6C and D respectively). Information regarding clone library origin and identification is presented in Table 3.2. Of 25 *dehI* clones sequenced, 8 grouped together in cluster I-1 (Figure 3.7), containing clones from day 39 of the 2MCPA-L test ($dehI_{M39\text{-}D}$ and $dehI_{M39\text{-}R}$ clone libraries; Table 3.2). All clones in this cluster showed high nucleotide sequence identity (≥ 98%). Another group of 8 clones from day 39 of the 2MCPA-L test ($dehI_{M39\text{-}D}$), again sharing high nucleotide sequence identity (≥ 98%), formed cluster I-2. Clusters I-1 and I-2 shared > 97% nucleotide sequence identity and were closely related to *DL-DEX* Mb from *Methylobacterium* sp. CPA1 (Omi et al., 2007) within subgroup B, as defined previously by Hill et al. (1999).

The remaining 9 clones formed cluster I-3, also within subgroup B. Cluster I-3 contained only clones generated from cDNA templates from day 39 of the 2MCPA-L test ($dehI_{M39\text{-}R}$; Table 3.2), with all except a single clone sharing 100% nucleotide sequence identity, and were closely related (84% nucleotide sequence identity) to $dehI_{AS1}$ and $dehI_{AS2}$ from *Xanthobacter* sp. AS1 and *Xanthobacter* sp. AS2 (Marchesi and Weightman, 2003b), respectively.

3.2.7 Diversity of group II dehalogenase genes in the DCA-L and 2MCPA-L tests

Clone libraries (Table 3.2) were prepared from *dehII* PCR products from both DCA-L and 2MCPA-L tests in order to assess the *dehII* diversity present in the starting population (day 0, libraries $dehII_{D0\text{-}D}$ and $dehII_{M0\text{-}D}$), to detect expression (transcription) of *dehII* genes (libraries $dehII_{D8\text{-}R}$ and $dehII_{M21\text{-}R}$), and to monitor any change in the *dehII* gene pool during the test ($dehII_{M39\text{-}D}$).

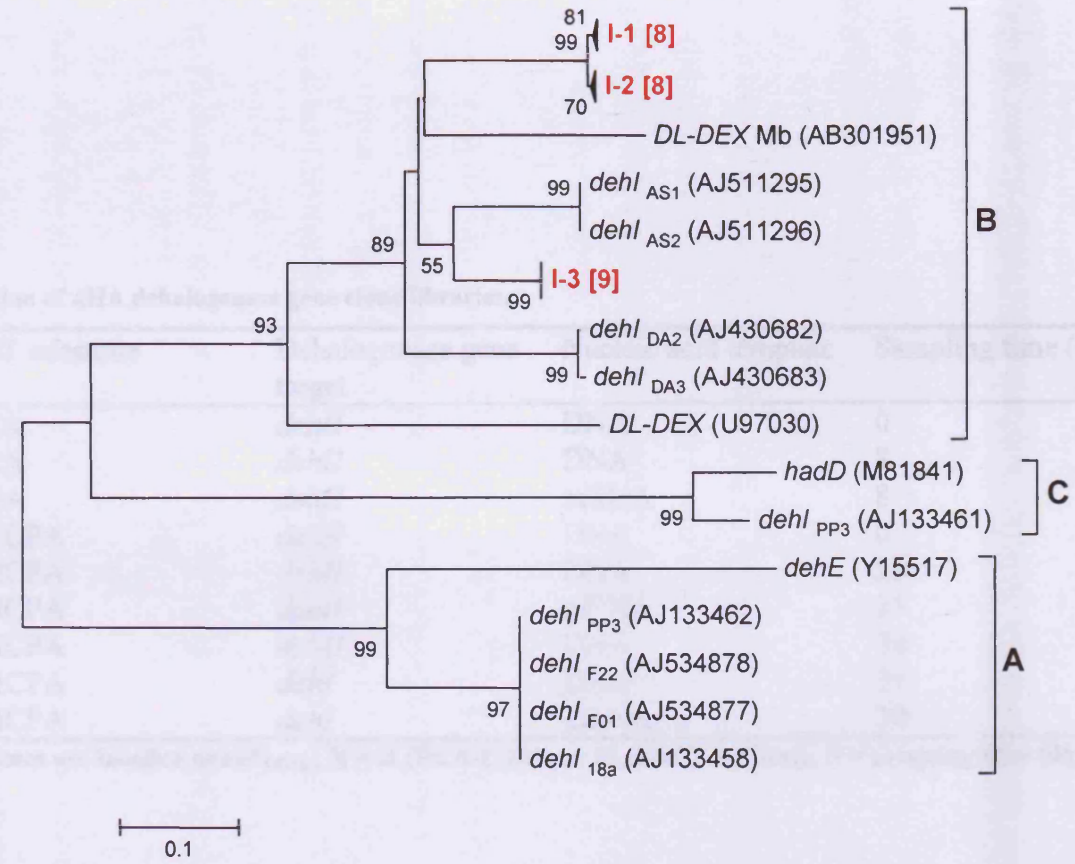

Figure 3.7 Dendrogram illustrating the phylogenetic relationship between *dehI* genes and their transcripts amplified from the 2MCPA-L test (coloured red), and references from the database. The tree was constructed from a final dataset of 219 nucleotides using the Neighbour-Joining method (Saitou and Nei, 1987) and the Jukes-Cantor algorhithm (Jukes and Cantor, 1969). Bootstrap values > 50, calculated from 1000 replicates, are shown as percentages at the nodes. Subgroupings were defined previously by Hill et al (1999). The scale bar represents the number of base substitutions per site. Accession numbers are included in the parenthesis and the number of clones present in a cluster is indicated in square brackets. Clusters I-1 to I-3 were grouped based on a nucleotide sequence identity of > 98%.

Table 3.2 Origin and identification of αHA dehalogenase gene clone libraries.

Test Identificaction	RBT substrate	Dehalogenase gene target	Nucleic acid template	Sampling time (days)[a]	Library name[b]
DCA-L	DCA	*dehII*	DNA	0	$dehII_{D0\text{-}D}$
DCA-L	DCA	*dehII*	DNA	8	$dehII_{D8\text{-}D}$
DCA-L	DCA	*dehII*	mRNA	8	$dehII_{D8\text{-}R}$
2MCPA-L	2MCPA	*dehII*	DNA	0	$dehII_{M0\text{-}D}$
2MCPA-L	2MCPA	*dehII*	DNA	21	$dehII_{M21\text{-}D}$
2MCPA-L	2MCPA	*dehII*	mRNA	21	$dehII_{M21\text{-}R}$
2MCPA-L	2MCPA	*dehII*	DNA	39	$dehII_{M39\text{-}D}$
2MCPA-L	2MCPA	*dehI*	DNA	39	$dehI_{M39\text{-}D}$
2MCPA-L	2MCPA	*dehI*	mRNA	39	$dehI_{M39\text{-}R}$

a. See Figure 1. b. Library names are labelled dehI/II$_{XN\text{-}Z}$; X = D (DCA-L test) or M (2MCPA-L test); N = sampling time (days); Z = Nucleic acid template used for PCR, D (DNA) or R (RNA).

Figure 3.8 shows the phylogenetic relationship between all *dehII* genes cloned and analysed in this study. The majority (85%; 61/72) of *dehII* clones analysed were assigned to subgroups A, C and D, as defined previously by Hill et al. (1999). The two largest clusters were II-1 (18 clones) and II-4 (23 clones), both in subgroup A (Figure 3.8). The II-1 cluster consisted entirely of clones from day 8 (Figure 3.1) of the DCA-L test (libraries $dehII_{D8\text{-}D}$ and $dehII_{D8\text{-}R}$, Table 3.2), sharing ≥ 99% nucleotide sequence identity, none of which were detected at the beginning of the enrichment (day 0). Another group of four clones (≥ 98% identity) from the $dehII_{D8\text{-}D}$ library, the II-2 cluster, formed the closest relative of the II-1 cluster. The II-4 cluster consisted of 23 clones from days 21 and 39 of the 2MCPA-L test: libraries $dehII_{M21\text{-}D}$, $dehII_{M21\text{-}R}$ and $dehII_{M39\text{-}D}$ (Table 3.2). Again, none of these clones were detected at the beginning of the test. All sequences in the II-4 cluster shared ≥ 99% nucleotide sequence identity and were most closely related (≥ 64%) to L-DEX (Nardi-Dei et al., 1994).

Ten clones from the starting population (day 0, Figure 3.1) formed a distinct group, consisting of clusters II-5, II-6 and II-7, most closely related to $dehII_{AS\text{-}gDNA\text{-}1}$ (Marchesi and Weightman, 2003b). All remaining clones from the starting population of both DCA-L and 2MCPA-L tests (denoted by an asterisk in Fig. 4B) were assigned to subgroup A.

Only clones from the two main clusters (II-1 and II-4, Figure 3.8) contained *dehII* gene clones with corresponding mRNA counterparts. The $dehII_{M21\text{-}G12\text{-}R}$ clone, which clustered within subgroup A (Figure 3.8), was a cDNA clone with no corresponding DNA counterpart detected in *dehII* gene clone libraries from either the DCA-L or 2MCPA-L tests. It shared 100% nucleotide sequence identity with the previously characterised *dehH2* (Kawasaki et al., 1992). The remaining 10 clones, all originating from DNA templates, were assigned within subgroups C and D.

3.2.8 Effect of storage of lyophilised activated sludge on RBT outcome

Following a period of storage at -20°C, the activity of lyophilised activated sludge was measured according to its ability to dechlorinate 2MCPA (Figure 3.9). During a 20 day testing period, little 2MCPA dechlorination was detected using 90 mg/l lyophilised activated sludge stored for 8 months. Following storage for 9 months, a sludge concentration 6 fold higher (180 mg/l) than recommended for the RBT was required to achieve total dechlorination of 2MCPA within (18 days) the 28-day period specified for the RBT.

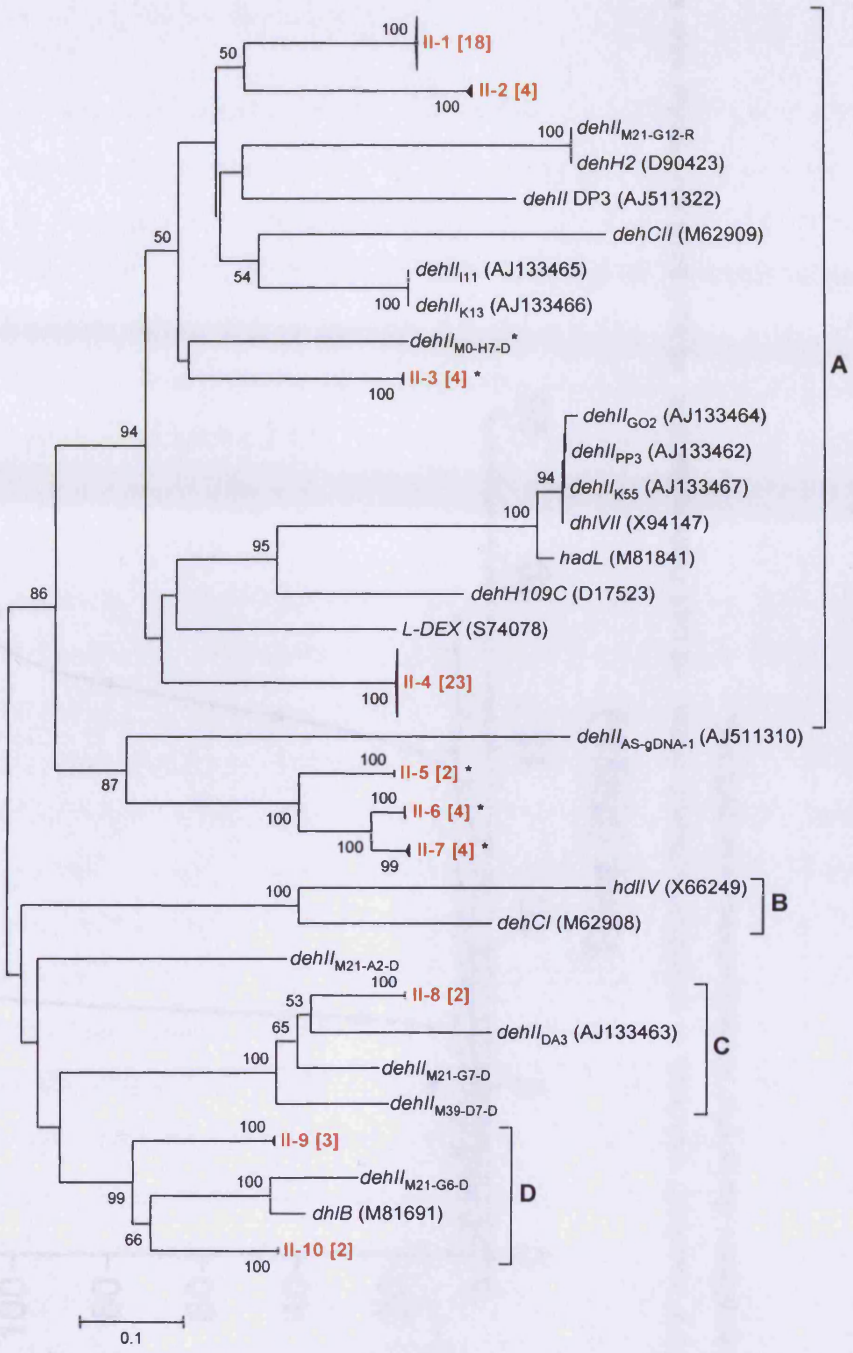

Figure 3.8 Dendrogram illustrating the phylogenetic relationship between *dehII* genes and their transcripts amplified from the DCA-L and 2MCPA-L tests (coloured red), and references from the database. The tree was constructed from a final dataset of 332 nucleotides using the Neighbour-Joining method (Saitou and Nei, 1987) and the Jukes-Cantor algorhithm (Jukes and Cantor, 1969). Bootstrap values > 50, calculated from 1000 replicates, are shown as percentages at the nodes. Subgroupings were defined previously by Hill et al (1999). The scale bar represents the number of base substitutions per site. Accession numbers are included in the parenthesis and the number of clones present in a cluster is indicated in square brackets. Individual clones from this study are identified as *dehII*$_{XN\text{-}Y\text{-}Z}$, where X = M (2MCPA enrichment) or D (DCA enrichment); N = sampling time (days); Y = clone ID; Z = nucleic acid template used for amplification, D (DNA) or R (RNA); see Table 3.2. Clones from the starting population (Day 0) are indicated by an asterisk. Clusters II-1 to II-10 were grouped based on a nucleotide sequence identity of > 98%.

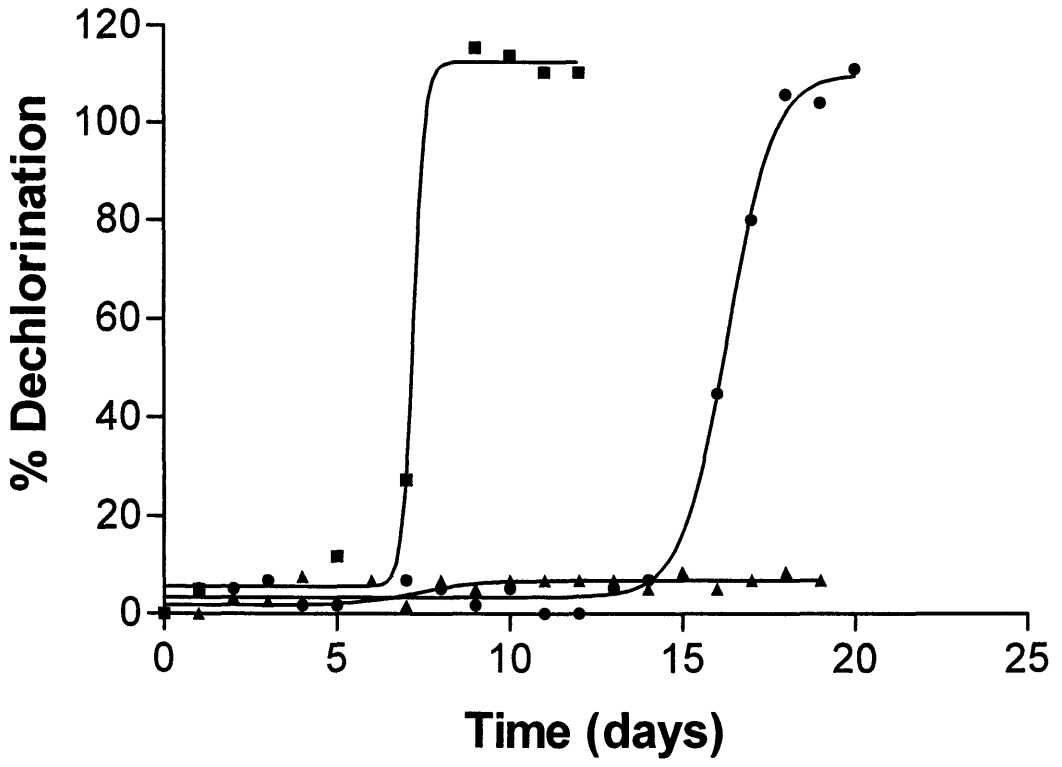

Figure 3.9 Degradation kinetics of 2MCPA inoculated with fresh or lyophilised activated sludge. 30 mg/l fresh sludge (■). 90 mg/l lyophilised sludge after 8 months of storage at -20°C(▲). 180 mg/l lyophilised sludge after 9 months of storage at -20°C (●).

3.3 Discussion

Model RBTs were conducted using the αHAs DCA and 2MCPA at concentrations (< 1 mM) far below those commonly used for the isolation of αHA degrading bacteria in the past (in the range of 5 – 30 mM; Slater et al., 1979; Tsang et al., 1988; Hasan et al., 1994; Dodds, 2003). For both test substrates, 100% dechlorination, which directly relates to complete substrate degradation and mineralisation (Slater et al., 1979), was detected (Figure 3.1), and shown to be associated with the enrichment of specific bacterial phylotypes (Figure 3.2 and 3.4), as well as the emergence and expression of αHA dehalogenase genes *dehI* and *dehII* (Figure 3.5 and 3.6).

DCA was passed as readily degradable in both DCA-F and DCA-L RBTs, with 70% substrate dechlorination (degradation) being observed well within the 28 day RBT limit (Figure 3.1). This was in agreement with other studies which have found DCA to be readily degraded by environmental inocula (Ellis et al., 2001). 2MCPA was rapidly dechlorinated in RBTs inoculated with fresh sludge, which is not surprising given the number of organisms reported to be capable of degrading this substrate (Slater et al., 1979; Janssen et al., 1985; Hill and Weightman, 2003). However, dechlorination of 2MCPA by lyophilised sludge proceeded much more slowly, and in two clearly distinct phases (Figure 3.1). This effect was highlighted by the RBT outcome, as the 2MCPA-F test would easily pass as readily degradable, whilst the 2MCPA-L test would not, having failed to achieve 70% dechlorination within the 28-day test limit. Since αHA dehalogenating organisms studied to date are almost exclusively *Proteobacteria* (Marchesi and Weightman, 2003b), this may be due to the comparatively low tolerance of Gram negative bacteria to lyophilisation (Miyamoto-Shinohara et al., 2006), resulting in lysis or loss of viability of important members of the activated sludge microbial community associated with 2MCPA degradation.

Both the DCA-L and 2MCPA-L test systems underwent dramatic changes in community structure, and dehalogenase gene complement, following exposure to αHAs. Such community re-structuring is common in bacterial populations exposed to xenobiotics, as demonstrated by Eichner et al. (1999) following phenol shocks, by Ayala-del-Rio et al. (2004) following phenol/trichloroethene shocks and by Bordenave et al. (2007) following contamination of pristine microbial mats by heavy

fuel oil. Following enrichment of lyophilised activated sludge using DCA as the test substrate, a *Ralstonia* like species (Figure 3.2A and B; Table 3.1) was selected, along with the expression of a *dehII* gene (Figure 3.5; cluster II-1, Figure 3.8). *Ralstonia* sp. have been previously reported as capable of degrading many different chlorinated pollutants, including trichlorophenol (Clément et al., 1995), various chlorophenoxyacetic acids (Pieper et al., 1988) and several different αHAs (Hill and Weightman, 2003). Previous reports have identified *Ralstonia* sp. containing *dehI* genes, as well as *dehII* (Hill and Weightman, 2003), though no *dehI* genes were detected at any stage of the DCA enrichment in this study (results not shown).

Dechlorination in the 2MCPA-L test was completed in two phases. Phase 1 (days 10 – 30, Figure 3.1) was associated with the enrichment of a *Methylibium petroleiphilum* like organism, as well as another unidentified phylotype (represented by bands M1/2 and M3/4 respectively; Figure 3.4 and Table 3.1) along with the expression of a *dehII* gene (Figure 3.6A and B). There have been no previous reports of *Methylibium* sp. containing active αHA dehalogenases, and although there are four putative dehalogenases annotated within the genome of *Methylibium petroleiphilum* PM1 (accession no NC_008825), none of these contain all of the amino acid residues required for dehalogenase activity in their derived amino acid sequence (Kurihara et al., 1995; Hisano et al., 1996; Li et al., 1998). It is probable that these genes are members of the broader haloacid dehalogenase (HAD) superfamily, which includes the DehII enzymes and several other hydrolases (Koonin and Tatusov, 1994). As in the DCA-L test, *dehII* expression, as detected by RT-PCR, occurred transiently during 2MCPA dechlorination, with peak expression at day 21 (Figure 3.6B). Clone libraries generated during peak *dehII* expression, $dehII_{M21\text{-}D}$ and $dehII_{M21\text{-}R}$ (Table 3.2), were dominated by a single group of clones (cluster II-4, Figure 3.8) suggesting this was the *dehII* responsible for the dechlorination observed in phase 1.

Phase 2 of 2MCPA dechlorination (days 36 – 44; Figure 3.1) was associated with the enrichment of a *Xanthobacter* sp. (bands M5/6; Figure 3.4 and Table 3.1) and *dehI* expression. The ability to degrade environmental pollutants appears to be a common trait in *Xanthobacter* spp. (Reij et al., 1995; Spiess et al., 1995; Tay et al., 1999; Torz et al., 2007), which have also been shown previously to degrade αHAs (Janssen et al., 1985; Marchesi and Weightman, 2003b). The majority of *dehI* clones present during

peak *dehI* expression (day 39, Figure 3.6D) formed clusters I-1 and I-2 (Figure 3.7), though only I-1 contained clones from mRNA templates ($dehI_{M39\text{-}R}$, Table 3.2) implicating this as the dehalogenase responsible for dechlorination during phase 2.

16S rRNA gene and cDNA profiles were generally similar to one another (65-94% similarity), with the notable exception of samples from days 0 and 6 of the DCA test. However, visible change within the bacterial community was slightly later in the DNA based analysis compared to the cDNA analysis. The transition from the population in phase 1 to that in phase 2 (i.e. the appearance of the *Xanthobacter* phylotype, bands M5 and M6, and the disappearance of the *Methylibium* phylotype, bands M1 and M2, Figure 3.4) was observed later in the DNA profile, as shown by the clustering of 2MCPA-L-36D with the samples from phase 1 (Figure 3.3). The greater sensitivity of rRNA based profiles has been demonstrated previously (Hoshino and Matsumoto, 2007), and is likely due to the greater persistence of DNA, compared to the more labile RNA – which have reported half lives as short as a few minutes (Rauhut and Klug, 1999; Selinger et al., 2003) – and the high variability of cellular RNA content, based on the metabolic condition of the organism; i.e. metabolically active cells tend to contain more ribosomes than starved or resting cells (Nomura et al., 1984; Aviv et al., 1996). Therefore, whilst RNA based profiling was more sensitive to changes in the bacterial population, both DNA and RNA profiles showed similar population changes during the model RBTs in this study.

Nine clones prepared from RNA templates at day 39 of the 2MCPA-L test ($dehI_{M39\text{-}R}$, Table 3.2) formed *dehI* cluster I-3 (Figure 3.7), with no DNA counterpart. This suggests either the enrichment of a second, highly expressed but low abundance dehalogenase within the system, or the presence of a silent dehalogenase gene (i.e. dehalogenase gene transcription is induced by the presence of the αHA substrate, though no functional enzyme is produced and consequently, the substrate can not be metabolised by this organism). Hill et al. (1999) described the presence of such a dehalogenase, $dehI^{\circ}$ from *Pseudomonas putida* PP3, which is transcribed in response to an αHA stimulus, though apparently not translated into an active dehalogenase due to a frame shift with respect to other genes in the *dehII* operon from which it is co-

transcribed. Other silent dehalogenases have also been reported by Köhler et al. (1998) and Tsang and Sam (1999).

The transient presence of dehalogenase transcripts observed in both the DCA-L (Figure 3.5B) and 2MCPA-L (Figure 3.6B and D) tests suggests the presence of inducible genes, transcribed in response to a specific αHA stimulus. This is consistent with previous studies which found dehalogenases to be inducible, commonly regulated by σ^{54}-dependent activators and associated with specific permease genes (Thomas et al., 1992b; Topping et al., 1995; van der Ploeg and Janssen, 1995; Dodds, 2003).

The biphasic dechlorination profile of 2MCPA seen during the 2MCPA-L test (Figure 3.1) may be explained by differences in the enantioselectivities of DehI and DehII enzymes. Whereas all previously characterised DehII enzymes are L-2MCPA specific, being unable to dechlorinate D-2MCPA (Klages et al., 1983; Tsang et al., 1988; Liu et al., 1994), DehI enzymes either act on both D- and L-2MCPA (Weightman et al., 1982; Liu et al., 1994; Schmidberger et al., 2008), or D-2MCPA only (Leigh et al., 1988). The detection of only *dehII* transcripts during phase 1 (day 21, Figures 3.1 and 3.6) suggests that L-2MCPA was degraded first by a DehII, leaving only D-2MCPA within the test system. The remaining D-2MCPA substrate would create a selection pressure for an organism containing a DehI, with activity towards D-2MCPA, which is in line with the observed emergence of *dehI* genes (detected by DNA and RNA analysis; Figure 3.6C and D) during phase 2 (Figure 3.1). The brief cessation of 2MCPA dechlorination at approx. 50% around day 30 (Figure 3.1) could therefore be explained by the enrichment of a *dehI* containing *Xanthobacter* sp. by day 36 (phase 2, Figure 3.1; bands M5/6 Figure 3.4). This represents a clear link between the community structure and function, and goes some way to explaining why 2MCPA failed the RBT in this instance.

Previous studies have demonstrated that group II dehalogenases predominate over the group I dehalogenases in the environment (Hill et al., 1999), a finding supported in this study where the starting populations (Day 0) contained detectable levels of *dehII* (Figure 3.5A and 3.6A) prior to any enrichment, but no detectable *dehI* (Figure 3.6C). The emergence of an active DehII, acting exclusively on the L-isomer, prior to that of

an active DehI, most likely capable of acting on both L- and D-isomers, is likely a reflection of this environmental abundance.

The 2MCPA-L test enriched a greater diversity of dehalogenases than the DCA-L test. Notwithstanding the presence of a *dehI* in the 2MCPA-L test (not detected at all during the DCA-L test), there was an apparent secondary enrichment within the *dehII* gene complement. During peak *dehI* expression at day 39 (phase 2), several novel *dehII* clones were detected, grouping in subgroups C and D (i.e. clusters II-9, II-10 and clone $dehII_{M39\text{-}D7\text{-}D}$, Figure 3.8), despite the lack of detectable *dehII* expression at this time. This may represent the co-enrichment of genetically linked *dehI* and *dehII* genes carried on the same host genomes. The presence of multiple αHA dehalogenases within the same genome is common in the well characterised bacteria producing these enzymes. *P. putida* AJ1 contains at least two active dehalogenases (Barth et al., 1992), *X. autotrophicus* GJ10 and *Pseudomonas* sp. CBS3 contain multiple dehalogenases (Klages et al., 1983; Janssen et al., 1985; Keuning et al., 1985) whilst *P. putida* PP3 contains two active and one silent dehalogenase (Slater et al., 1979; Weightman et al., 1979).

The *dehII* Clusters II-5, II-6 and II7 (Figure 3.8) constituted the closest relative of clone $dehII_{AS\text{-}gDNA\text{-}1}$, representing an uncultivated lineage (Marchesi and Weightman, 2003b); thus, this clade was not closely related to any *dehII* sequence from a cultivated isolate. Marchesi and Weightman (2003b) putatively identified $dehII_{AS\text{-}gDNA\text{-}1}$ as a member of a novel dehalogenase subgrouping, based on the presence of conserved amino acid residues essential for catalysis (Kurihara et al., 1995; Hisano et al., 1996) and the hydrophobic pocket of the enzyme (Li et al., 1998) in its derived amino acid sequence. The 10 clones in the II-5/II6/II-7 clade also contain these conserved residues, supporting their identification as a novel dehalogenase subgroup. However, without detectable *dehII* expression at this stage of the enrichment, and given the placement of DehII enzymes within the much broader haloacid dehalogenase (HAD) superfamily (Koonin and Tatusov, 1994), their identification as *bona fide* dehalogenases remains tentative.

Lyophilised activated sludge gave comparable RBT results to those obtained using fresh sludge inocula when DCA was the test substrate; however degradation of

2MCPA in RBTs inoculated with lyophilised activated sludge were quite different (Figure 3.1). The 2MCPA degradative capability of lyophilised activated sludge was further reduced following storage at -20°C over a period of several months (Figure 3.9). Little 2MCPA dechlorination was observed using stored lyophilised sludge at 3 times (90 mg/l) the recommended RBT inoculum concentration (30 mg/l). Even at 6 times (180 mg/l) the recommended RBT inoculum concentration, 2MPCA dechlorination took twice as long as in the fresh sludge test (Figure 3.9). The diminished activity may have been due to deterioration of the lyophilised sludge, caused by some facet of the storage conditions used since samples were simply stored in microfuge tubes. Previous studies have shown that samples stored in glass vials under vacuum, or in the presence of inert gas, show increased "shelf life" compared to those stored in air (Bozoğlu et al., 1987; Morgan et al., 2006). These results suggest that lyophilisation of complex environmental inocula, such as activated sludge, may have some utility as a standard inoculum for biodegradation testing. However, further study is required to investigate the stability of stored lyophilised sludge. The addition of specific protective agents, such as carbohydrates (e.g. sucrose or trehalose; Israeli et al., 1993; Leslie et al., 1995), or polymers such as polyvinylpyrrolidone (Lodato et al., 1999), prior to lyophilisation may serve to stabilise the activated sludge during storage. Alternative methods of drying may also be worth investigating, such as spray drying, which has been used previously for drying and storing bacteria (Gardiner et al., 2000; Silva et al., 2002). As lyophilised activated sludge could not be stably maintained for the duration of this study, all other RBTs conducted in this thesis used freshly collected activated sludge.

This study further emphasises the drawbacks of conventional *in vitro* cultivation based methods as a direct model for environmental processes *in situ*. Previous studies focus predominantly on the isolation of degraders from environmental samples following successive rounds of sub-culturing (Tsang et al., 1988; Hasan et al., 1994). Sub-cultures are often taken after complete substrate degradation, and as such, risk missing intermediate stages and succession during community acclimation. For instance, end point sampling of the 2MCPA-L test in this study would have missed the succession of the *Methylybium* phylotype by the *Xanthobacter* phylotype. Using a combination of 16S rRNA profiling and catabolic gene analysis, it has been possible

to relate community structure and catabolic gene selection to a functional process – dechlorination – which represents RBT outcome. This highlights the utility of molecular genetic techniques, making them essential tools for successfully interpreting the outcome of RBTs.

4. The Effects of Varying Inoculum Concentration on Biodegradation of 2-Monochloropropionic Acid (2MCPA) In a Model Ready Biodegradation Test (RBT) System

4.1 INTRODUCTRION

The lack of regulation regarding inoculum source and pre-treatment has been largely blamed for inconsistent ready biodegradation test (RBT) results, however, inoculum size has received little attention. Inoculum size may conceivably affect RBT outcome in several ways; by regulating the amount of additional degradable organic substrates added, the level of protozoal predation, and especially by controlling the presence and number of specific degraders within the test system. Each of these factors could potentially affect a chemical's RBT result.

Even at standard RBT inoculum concentration of 30 mg/l, it is supposed that approximately 15 mg/l of additional carbon is added to the test system in the inoculum, almost as much as is available from the test compound (Birch and Fletcher, 1991). The presence of other readily degradable carbon sources, added to the test system along with the inoculum, may enhance the degradation of the test compound due to cometabolism. Alternatively, substrate degradation may be delayed due to the preferential utilisation of the additional readily degradable carbon sources (Becker et al., 2006). Most recent studies investigating the effect of substrate mixtures on microbial growth are restricted to pure cultures (Kar et al., 1997; Reardon et al., 2000; Lee et al., 2003), whilst little is currently understood regarding the effects of multiple substrate utilisation by mixed cultures (Becker et al., 2006).

Several studies have demonstrated that protozoal predation can increase the lag phase during biodegradation of xenobiotics. The application of cycloheximide, a potent inhibitor of eukaryotic organisms, to biodegrading systems served to reduce lag phases during *p*-nitrophenol degradation (Wiggins et al., 1987; Zaidi et al., 1996), suggesting that reduced predation allowed more rapid proliferation of specific degrading organisms.

Another important factor affecting chemical biodegradation is the presence of specific degrading organisms within the system, capable of accomplishing the task.

Significantly, this population may not be numerically dominant within the inoculum, affecting their ability to form a strong degrading population (Ingerslev et al., 2000). Franklin et al. (2001) showed that serial dilution and re-growth of activated sludge samples significantly altered the community structure and diversity in the higher dilutions, which in turn affected the community organisation and function, thus the inoculum size is critical in determining how the community will function.

Each of these factors may affect the outcome of the RBT differently. For instance, whilst a larger inoculum size would increase the concentration of specific degraders within the system – potentially advancing the onset and enhancing the rate of biodegradation – it would also increase the amount of additional carbon and protozoal predators present, potentially slowing the onset of biodegradation. The acclimation of microbial communities to degrade xenobiotic compounds is normally studied by the characterisation of isolated organisms (Wiggins et al., 1987; Liu et al., 2002; Kerr and Marchesi, 2006). Cultivation-independent, nucleic acid based methods provide an alternative approach, where a much greater proportion of the microbial community is accessible than by cultivation dependent methods (Wagner et al., 1993).

2-Chloropropionic acid (2MCPA) has been used in the past as a herbicide and as a chemical precursor for the synthesis of phenoxypropionic acid herbicides (Dodds, 2003; Kurihara and Esaki, 2008), and it is readily degraded by many bacteria (Slater et al., 1979; Janssen et al., 1985). In Chapter 3 it was demonstrated that 2MCPA was readily degradable under normal RBT conditions (using fresh sludge at 30 mg/l), though under certain conditions – presumably following the perturbation of a specific degrading population – 2MCPA degradation required the separate enrichment of two different organisms and failed the RBT. This study aimed to investigate what causes a readily degradable compound like 2MCPA to fail the RBT, by using a standard (30 mg/l), an intermediate (3 mg/l) and a low (0.3 mg/l) inoculum size, with particular attention to the bacterial community change taking place, and the specific degrading populations.

4.2 RESULTS

4.2.1 2MCPA degradation

At the standard RBT inoculum concentration (30 mg/l), 2MCPA was totally dechlorinated just after day 8 in all three replicate flasks, following an initial lag of around 6 days (Figure 4.1A). All three replicates (30A, 30B and 30C) fell well within the RBT pass level.

At 3 mg/l activated sludge (Figure 4.1B), both 3A and 3B showed biphasic curves. Dechlorination of 2MCPA in 3A began after a 3 day lag, and continued until day 7, where dechlorination ceased for approximately 6 days, before continuing to complete dechlorination at day 14. Chloride release began after a 5 day lag in 3B, and continued until day 8 where it plateaued briefly for around 1 day, before reaching total dechlorination at day 12. Test 3C showed total dechlorination of 2MCPA in 7 days following a lag phase of 3 days. All three replicates achieved the 70% pass level within the time limits of the RBT.

Dechlorination of 2MCPA in the 0.3 mg/l tests (Figure 4.1C) was preceded by a lag of 7 and 8 days in 0.3A and C respectively, but 0.3B had a longer lag of around 15 days. Dechlorination ceased after reaching 50% in all three replicates (days 9, 19 and 12 for 0.3A, B and C, respectively) and was followed by a lengthy plateau of between 15 and 34 days. All three replicates were totally dechlorinated at days 46, 67 and 107 for 0.3A, 0.3B and 0.3C, respectively, though none reached the 70% pass mark within the 28 day time limit of the RBT.

4.2.2 DGGE community profiling

Following the onset of substrate dechlorination, test 30A was dominated by a single phylotype (band no 30-1; Figure 4.2A; Table 4.1), closely related to a *Herbaspirillum* like member of the *Betaproteobacteria*. An *Acidovorax* sp. (band no 30-2) was also present during active substrate dechlorination, though it was far less prominent than the *Herbaspirillum* (30-1). Test 30B (Figure 4.2B) showed the enrichment of two phylotypes, not present in the starting population. A *Pseudomonas* like phylotype (band 30-3) dominated the profile in this test, whilst a fainter band (30-4), identical to the *Herbaspirillum* in 30A (band 30-1), was also present. The *Pseudomonas*

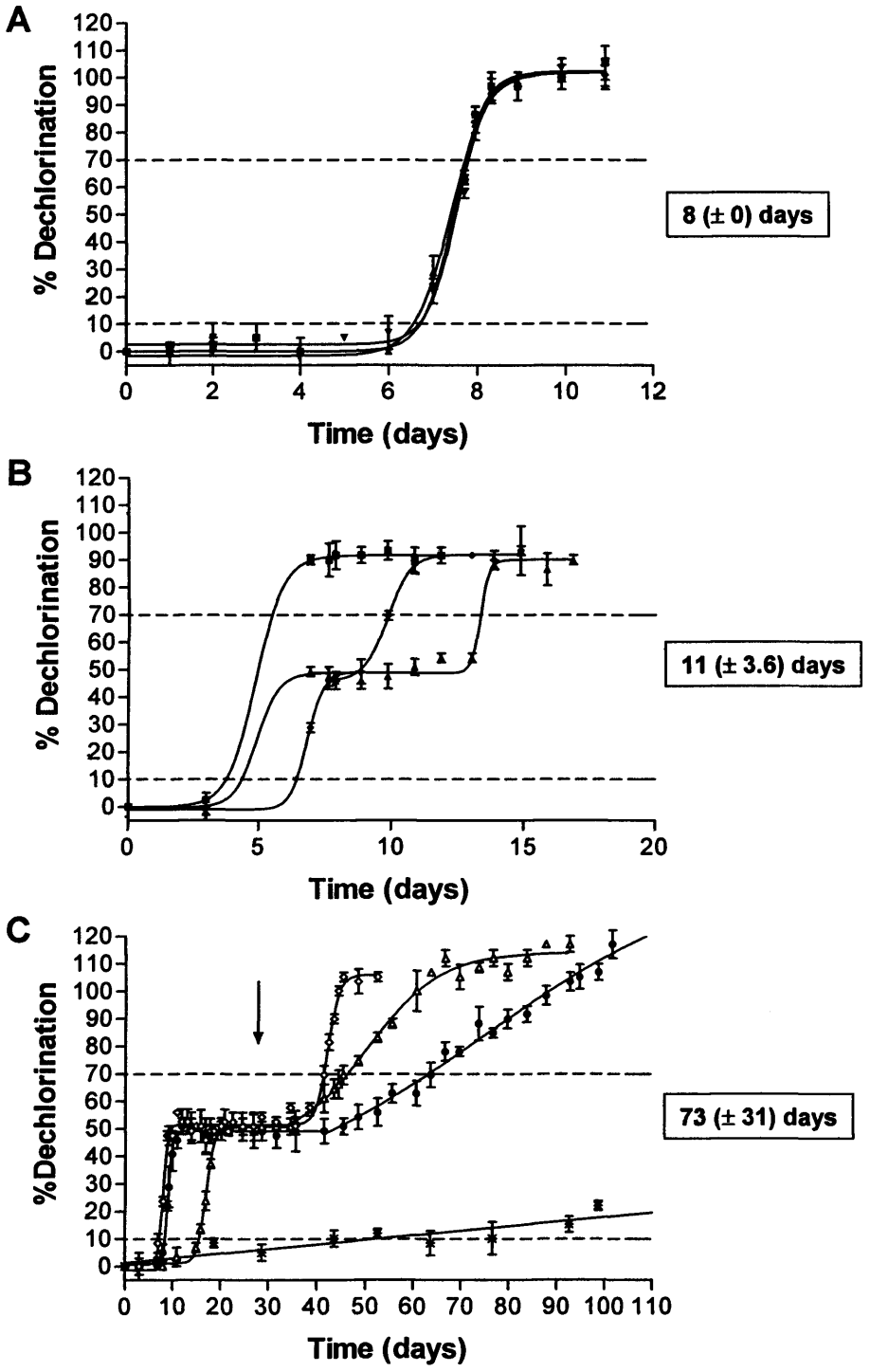

Figure 4.1 Degradation kinetics of 2MCPA with varying inoculum sizes. (A) 30 mg/l activated sludge; 30A = ■, 30B = ▲, 30C = ▼. (B) 3 mg/l activated sludge; 3A = ▲, 3B = ◆, 3C = ■. (C) 0.3 mg/l activated sludge; 0.3A = ◇, 0.3B = △, 0.3C = ●. ✳ = abiotic control. Broken horizontal lines represent biodegradation start point (10%) and pass level (70%). Arrow indicates the 28 day cut-off for a RBT pass. Error bars represent the standard error of the mean (SEM) (n = 3). Boxed values represent the mean time taken to achieve 100% dechlorination (n = 3; ± standard deviation).

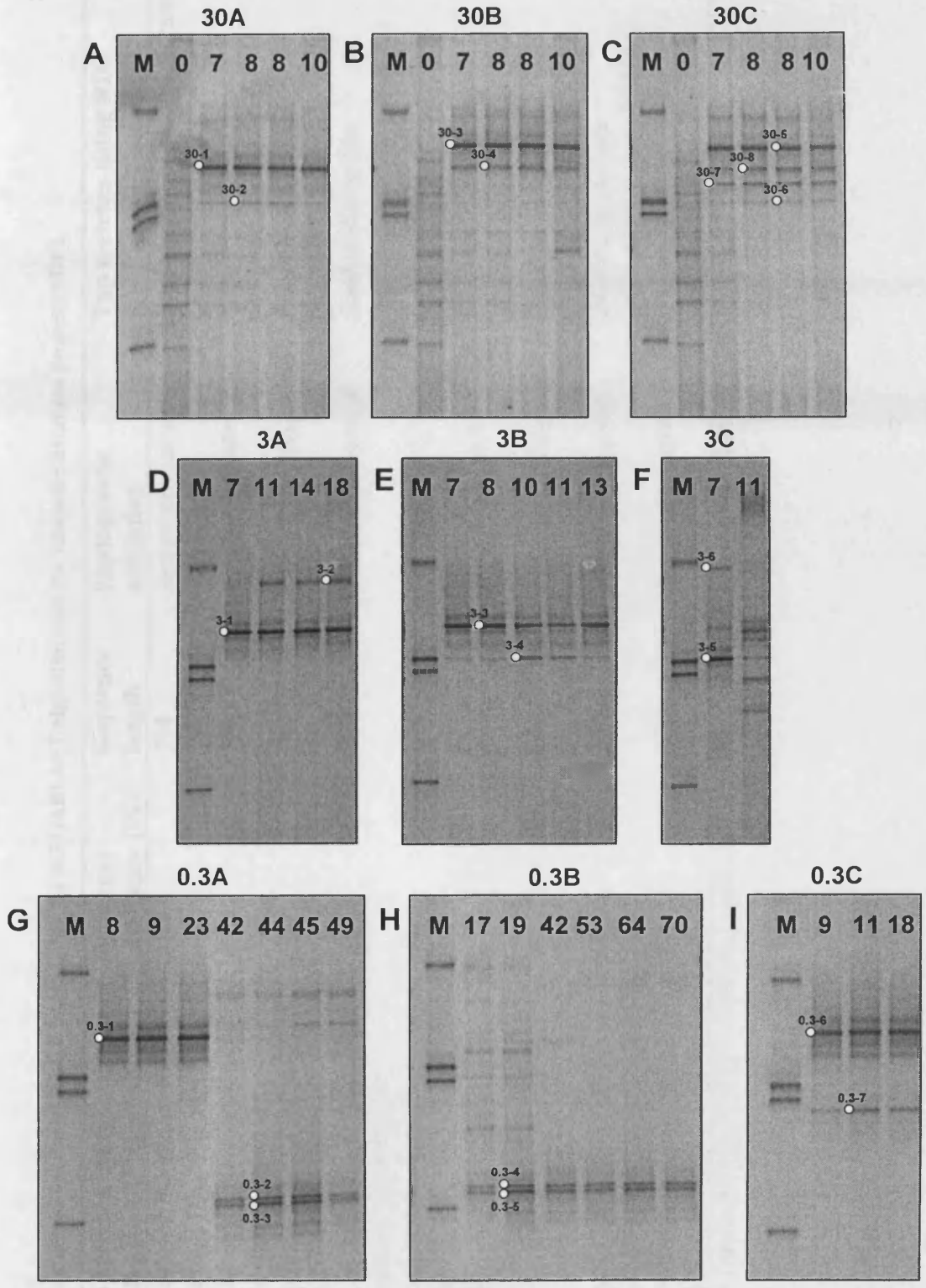

Figure 4.2 DGGE of the PCR amplified 16S rRNA gene from selected time points during 2MCPA RBTs at different activated sludge concentrations. (A) 30A test. (B) 30B test. (C) 30C test. (D) 3A test. (E) 3B test. (F) 3C test. (G) 0.3A test. (H) 0.3B test. (I) 0.3C test. Numbers above each lane represent time (days) of sampling (see Figure 4.1), M = marker. Open circles represent bands excised for sequencing. Numbers above each panel represent the RBT identifier.

Table 4.1 Closest sequence matches to excised DGGE bands using the MEGABLAST algorithm and the ribosome database project (RDP).

Band identification	Top matches using MEGABLAST algorhithm	Sequence similarity (%)	Sequence length	Phylogenetic affiliation	Top matches using RDP
30-1; 30-4; 3-1; 3-3;	Uncultured *Burkholderiales* bacterium (EU572350)	100	194	*Betaproteobacteria*	*Herbaspirillum autotrophicum* (AB074524)
30-2; 30-6; 3-4; 3-5	Uncultured *Proteobacterium* clone (GQ502601)	99	194	*Betaproteobacteria*	*Acidovorax temperans* (AF078766)
30-3; 30-5	*Pseudomonas fluorescens* strain Gd2F (GU391472)	100	194	*Gammaproteobacteria*	*Pseudomonas fragi* (AF094733)
30-7[a]; 30-8; 0.3-1	Uncultured *Oxalobacteraceae* bacterium (AM940769)	99	194	*Betaproteobacteria*	*Janthinobacterium agaricidamnosum* (Y08845)
3-2	*Pseudomonas* sp. J465 (GQ370386)	99	194	*Gammaproteobacteria*	*Pseudomonas peli* (AM114534)
3-6	*Pseudomonas* sp. I-Bh4-8 (FN555395)	100	194	*Gammaproteobacteria*	*Pseudomonas panacis* (AY787208)
0.3-2; 0.3-3; 0.3-4; 0.3-5	*Starkeya* sp. ly5 (FJ577972)	99	169	*Alphaproteobacteria*	*Starkeya koreensis* (AB166877)
0.3-6	*Duganella* sp. HMD2171 (GQ354570)	99	194	*Betaproteobacteria*	*Janthinobacterium agaricidamnosum* (Y08845)
0.3-7	*Hydrogenophaga* sp. BALT-12-S2.1 (FM998722)	99	194	*Betaproteobacteria*	*Hydrogenophaga palleronii* (AF078769)

a. Band 30-7 differed from bands 30-8 and 0.3-1 by a single nucleotide; otherwise bands grouped together shared 100% sequence identity.

phylotype present in test 30B also dominated in test 30C (band 30-5), though it was accompanied by several other phylotypes (Figure 4.2C). Again, the *Acidovorax* sp. from 30A was present (30-6), whilst band 30-7 was most similar to *Janthinobacterium* sp. and an uncultivated *Oxalobacteraceae* bacterium, neither of which appeared in any of the other 30 mg/l tests. Band 30-8 was almost identical to band 30-7 (1 nucleotide difference), despite apparently co-migrating with bands 30-1 and 30-4. The closest sequence matches for all bands labeled in Fig. 4.2 are shown in Table 4.1.

The 16S rRNA gene was below the level of detection by PCR at day 0 for all of the 3 mg/l tests, and at day 3 for the 3A and 3C tests. DGGE community profiles from tests 3A and 3B (Figure 4.2 D & E, respectively) were initially dominated by bands 3-1 and 3-3, which were identical to the *Herbaspirillum* phylotype present in 30A and 30B (bands 30-1 and 30-4). This was joined by a *Pseudomonas* phylotype (band 3-2) at day 11 in 3A, whilst the *Acidovorax* phylotype present in 30A and 30C was also present in 3B, appearing most strongly at day 10 (band 3-4). Test 3C was dominated by band 3-5 (Figure 4.2F), which matched the *Acidovorax* sp. present in 30A, 30C and 3B, whilst a *Pseudomonas* phylotype was also present (band 3-6).

Replicate tests at 0.3 mg/l activated sludge showed the simplest DGGE profiles. A *Janthinobacterium*, identical to band 30-8, and differing from band 30-7 by a single nucleotide dominated the early stages of tests 0.3A (Figure 4.2G, days 8-23 – band 0.3-1). This was replaced at day 42 by two separate, but apparently identical, bands (0.3-2 and 0.3-3) identified as a *Starkeya* like phylotype, which became more prominent until day 45, before fading by day 49. The same *Starkeya* phylotype dominated throughout test 0.3B (Figure 4.2H, bands 0.3-4 and 0.3-5) though a few other weak and unidentifiable bands were also present during days 17 and 19. Test 0.3C contained a dominant phylotype belonging to the *Betaproteobacteria* (Figure 4.2I, band 0.3-6) with similarities to both *Janthinobacterium* and *Duganella* species. Another *Betaproteobacterium*, a *Hydrogenophaga* like sp., was also present during days 9-18 (band 0.3-7). No 16S rRNA gene PCR products were detected at any time point sampled after day 18 of 0.3C.

4.2.3 Presence and expression of α-HA dehalogenase genes

In addition to profiling the bacterial community changes that occurred during biodegradation of 2MCPA by 16S rRNA gene PCR-DGGE, the presence or absence of dehalogenase genes *dehI* and *dehII* were determined using the PCR primers designed by Hill et al. (1999). This analysis included RT-PCR with RNA extracts from selected time points to detect *dehI* and *dehII* gene expression. However, as only the shorter of the two *dehI* PCR products was detectable in the majority of the test systems, all results reported hereafter refer to the shorter (230 bp) *dehI* PCR product.

The *dehI* PCR products were not detectable at day 0 of the 30 mg/l tests, though they were detected at all time points sampled following the onset of dechlorination (Figure 4.3A, days 7-10). The *dehII* PCR products were detectable at every time point sampled in the 30 mg/l tests. RT-PCR revealed dehalogenase transcripts in all the 30 mg/l tests. Transcripts of both *dehI* and *dehII* were first detected at day 7, and *dehI* transcripts remained at a detectable level in all subsequently sampled time points, though *dehII* transcripts had dropped below the level of detection by day 10 (Figure 4.3A).

Neither *dehI* nor *dehII* was detectable at day 0 in any of the 3 mg/l tests (Figure 4.3B), and PCR products for both genes were detectable only after the onset of dechlorination at day 7 for all except 3A, where *dehI* was only clearly detected at day 11. Both *dehI* and *dehII* remained at detectable levels for all other samples analysed. Dehalogenase transcripts were also detected within the 3 mg/l test systems. In 3A, *dehII* transcripts were detectable only at day 7, where as *dehI* transcripts were detectable at days 11 and 14, but were undetectable by day 18. Transcripts from *dehI* appeared at day 7 in test 3B, and remained at detectable levels for all other time points sampled, and *dehII* transcripts were also present in this test from day 7 until day 10, but were not detectable thereafter. The *dehI* transcripts were detectable only at day 7 in test 3C, but no *dehII* transcripts were detected at any stage of the test.

Again, neither dehalogenase was present at a detectable level in any of the 0.3 mg/l tests prior to the onset of substrate dechlorination. Dehalogenases were detectable at days 8, 17 and 9 for tests 0.3A, 0.3B and 0.3C, respectively. Initially, only *dehII* was

A

30A	0	7	8	8	10
dehI	-	+	+	+	+
mRNA	-	+	+	+	+
dehII	+	+	+	+	+
mRNA	-	+	+	+	-

30B	0	7	8	8	10
dehI	-	+	+	+	+
mRNA	-	+	+	+	+
dehII	+	+	+	+	+
mRNA	-	+	+	+	-

30C	0	7	8	8	10
dehI	-	+	+	+	+
mRNA	-	+	+	+	+
dehII	+	+	+	+	+
mRNA	-	+	+	+	-

B

3A	0	3	7	11	14	18
dehI	-	-	-	+	+	+
mRNA	-	-	-	+	+	-
dehII	-	-	+	+	+	+
mRNA	-	-	+	-	-	-

3B	0	7	8	10	11	13
dehI	-	+	+	+	+	+
mRNA	-	+	+	+	+	+
dehII	-	+	+	+	+	+
mRNA	-	+	+	+	-	-

3C	0	3	7	11
dehI	-	-	+	+
mRNA	-	-	+	-
dehII	-	-	+	+
mRNA	-	-	-	-

C

0.3A	0	8	9	23	42	44	45	49
dehI	-	-	-	-	+	+	+	+
mRNA	-	-	-	-	+	+	+	+
dehII	-	+	+	+	+	+	+	+
mRNA	-	+	+	-	-	-	-	-

0.3B	0	11	17	19	42	53	64	70
dehI	-	-	+	+	+	+	+	+
dehII	-	-	+	+	+	+	+	+

0.3C	0	9	11	18	42	70	93	115
dehI	-	-	-	-	-	-	-	-
dehII	-	+	+	+	-	-	-	-

Figure 4.3 Presence or absence of *dehI* and *dehII* genes and their transcripts at selected time points. Samples were taken from triplicate tests conducted at (A) 30 mg/l, (B) 3 mg/l and (C) 0.3 mg/l activated sludge inoculum. Red squares and (-) represent absence of (RT)-PCR products. Green squares and (+) represent presence of (RT)-PCR products. Numbers above each column represent sampling time (days) relating to Figure 4.1. Test identification is shown in the upper left corner of each punnet.

detectable in test 0.3A, first appearing at day 8, followed by *dehI* which appeared at day 42. Both *dehI* and *dehII* remained at detectable levels in all other time points sampled in 0.3A. The *dehII* transcripts were only detected in test 0.3A at days 8 and 9, whilst *dehI* transcripts were present from day 42, and in all subsequently sampled time points. Both *dehI* and *dehII* appeared concurrently at day 17 in test 0.3B, and remained at a detectable level thereafter. The *dehII* gene was first detectable at day 9 in test 0.3C and persisted until day 18, before dropping below the level of detection by day 42 and remaining absent for the remainder of the test. *dehI* was not detectable at any stage in 0.3C. No dehalogenase transcripts were detected in either 0.3B or 0.3C.

4.2.4 Variability within the *dehI* gene pool

Group I dehalogenase gene and cDNA libraries were prepared from dehalogenase PCR products to assess the dehalogenase gene diversity at selected time intervals throughout each test. Appropriate time points were selected in order to assess the dehalogenase diversity in the unacclimated sludge (day 0), as well as in acclimated sludge during active substrate dechlorination. Where possible, time points from replicate tests at the same sludge concentration were pooled and analysed as a single sample. A summary of all dehalogenase gene libraries constructed and analysed in these experiments, indicating the time points sampled (see also Figure 4.1) is shown in Table 4.2. The tree topology from Figure 4.4 was confirmed using either nucleotide sequence or amino acid sequence data in the phylogenetic analysis.

All of the *dehI* clones analysed were assigned to one of four distinct clusters by phylogenetic analysis (Figure 4.4). The *dehI* cluster 1 included clones from both DNA and cDNA templates across four different libraries, including the 30-8 and 30-8-R libraries, the 3A-14 library and a single clone from the 3B-11 library (Figure 4.4; Table 4.2). All clones from cluster 1 shared > 99% sequence identity, and were virtually identical (> 99%) to *dehI°* from *Pseudomonas putida* PP3 (Hill et al., 1999). The *dehI* cluster 2 (Figure 4.4) was the most prevalent *dehI* across all tests, being present in the 30-8 and 30-8-R, the 3B-11 and 3B-11-R and 3C-7 and 3C-7-R libraries, as well as from the 3A-14 library (Figure 4.4; Table 4.2 – no cDNA based library was prepared from 3A day 14). The *dehI* clusters 1 and 2 grouped within subgroup C, as defined by Hill et al. (1999). All *dehI* clones from the 0.3B-64 library were identical, except for a single clone which differed by a single nucleotide, and

Table 4.2 Origin and identification of αHA dehalogenase gene libraries from this study.

Library name[a]	Test ID	Dehalogenase gene target	Sampling time (days)[b]	Nucleic acid template
$dehII_{30\text{-}0}$	Pooled 30A, 30B and 30C	*dehII*	0	DNA
$dehI_{30\text{-}8}$	Pooled 30A, 30B and 30C	*dehI*	8	DNA
$dehI_{30\text{-}8\text{-}R}$	Pooled 30A, 30B and 30C	*dehI*	8	cDNA
$dehII_{30\text{-}8}$	Pooled 30A, 30B and 30C	*dehII*	8	DNA
$dehII_{30\text{-}8\text{-}R}$	Pooled 30A, 30B and 30C	*dehII*	8	cDNA
$dehII_{3A\text{-}7}$	3A	*dehII*	7	DNA
$dehI_{3A\text{-}14}$	3A	*dehI*	14	DNA
$dehII_{3A\text{-}14}$	3A	*dehII*	14	DNA
$dehII_{3B\text{-}8}$	3B	*dehII*	8	DNA
$dehI_{3B\text{-}11}$	3B	*dehI*	11	DNA
$dehI_{3B\text{-}11\text{-}R}$	3B	*dehI*	11	cDNA
$dehII_{3B\text{-}11}$	3B	*dehII*	11	DNA
$dehI_{3C\text{-}7}$	3C	*dehI*	7	DNA
$dehI_{3C\text{-}7\text{-}R}$	3C	*dehI*	7	cDNA
$dehII_{3C\text{-}7}$	3C	*dehII*	7	DNA
$dehII_{3\text{-}R}$	Pooled 3A and 3B	*dehII*	7; 8	cDNA
$dehII_{0.3A\text{-}9}$	0.3A	*dehII*	9	DNA
$dehII_{0.3A\text{-}45}$	0.3A	*dehII*	45	DNA
$dehI_{0.3A\text{-}45}$	0.3A	*dehI*	45	DNA
$dehII_{0.3B\text{-}19}$	0.3B	*dehII*	19	DNA
$dehII_{0.3B\text{-}64}$	0.3B	*dehII*	64	DNA
$dehI_{0.3B\text{-}64}$	0.3B	*dehI*	64	DNA
$dehII_{0.3C\text{-}11}$	0.3C	*dehII*	11	DNA

a. Library names are labelled dehI/II$_{CL\text{-}N\text{-}R}$, where C = sludge concentration (mg/l), L = test identification (A, B, C or no designation if pooled samples used), N = sampling time (days); R = cDNA template used for amplification (if absent, DNA was used as a template). For example dehI$_{3B\text{-}11\text{-}R}$ was a *dehI* gene sampled from test 3B on day 11, amplified from a cDNA template. b. Refer to Figure 4.1.

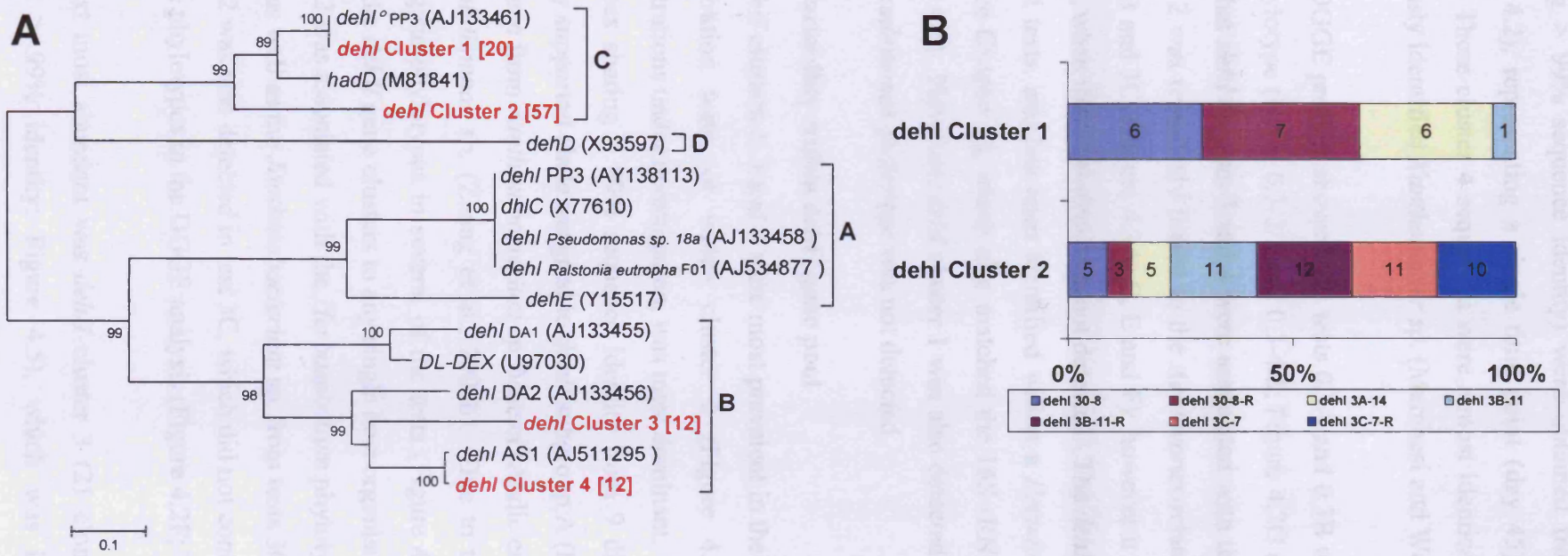

Figure 4.4 Unrooted dendrogram illustrating the phylogenetic relationship between *dehI* genes and their transcripts amplified in this study (coloured red), and references from the database. (A) The tree was constructed from a final dataset of 238 nucleotides using the Neighbor-Joining method (Saitou and Nei, 1987) and the Jukes-Cantor algorithm (Jukes and Cantor, 1969). Bootstrap values > 50, calculated from 1000 replicates, are shown as percentages at the nodes. Subgroups were defined previously by Hill et al. (1999). The scale bar represents the number of base substitutions per site. Accession numbers are included in the parenthesis and the number of clones present in a cluster is indicated in square brackets. (B) Breakdown of the composition of *dehI* clusters 1 and 2. Bars represent fraction of the cluster occupied by each library. Numbers of clones represented by each bar are also displayed.

were assigned to cluster 3 within subgroup B. All clones assigned to *dehI* cluster 4 (sharing > 99% sequence identity) were obtained from a single library, 0.3A-45 (Table 4.2), representing a single time point (day 45) from the 0.3A test (Figure 4.1C). These cluster 4 sequences were almost identical (> 99%) to $dehI_{AS1}$ from a previously identified *Xanthobacter* sp. (Marchesi and Weightman, 2003b).

Since DGGE profiles showed that tests 0.3A and 0.3B were dominated by a *Starkeya* like phylotype (bands 0.3-2/3 and 0.3-4/5; Figure 4.2G and H, respectively), it seems likely that *dehI* clusters 3 and 4 were associated with this organism. Similarly, *dehI* cluster 2 was tentatively linked to the *Acidovorax*-related phylotype present in 30A, 30C, 3B and 3C, (Figure 4.2A, C, E and F); however it was also present at day 14 in test 3A, when this phylotype was not detected. The *dehI* cluster 1 was linked with the 30 mg/l tests, and has been identified within a *Pseudomonas* sp. isolated from test 30C (see Chapter 6), which also matched the 16S rRNA sequence for bands 30-1/5 (Figure 4.2). However, *dehI* cluster 1 was also detected in tests 3A and 3B, in which this *Pseudomonas* phylotype was not detected.

4.2.5 Variability within *dehII* gene pool

The *dehII* clusters 1, 2 and 3 were most prevalent in the high and intermediate sludge concentration tests, of which cluster 2 (Figure 4.5), detected at all sludge concentrations under investigation, was most dominant. The *dehII* cluster 2 contained 73 clones sharing > 99% sequence identity from 9 different libraries, and gave a strongly supported clade assigned to *dehII* subgroup A (Hill et al., 1999), along with a *dehII* gene from *Janthinobacterium* sp. Marsei (Audic et al., 2007) and $dehII_{GM2}$ from a *Herminiimonas* sp. (Zhang et al., 2009b). Due to the presence of multiple 16S rRNA gene phylotypes in several of the tests (Figure 4.2), it was difficult to assign any of the *dehII* gene clusters to any single host organism with confidence. The *dehII* cluster 2 was associated with the *Herbaspirillum* phylotype from tests 30A and B, 3A and B, as well as the *Janthinobacterium* sp. from tests 30C and 0.3A. However, *dehII* cluster 2 was also detected in test 3C, which did not contain detectable levels of either of these phylotypes in the DGGE analysis (Figure 4.2F; Table 4.1).

The next most abundant was *dehII* cluster 3 (21 clones from 6 different libraries sharing > 99% identity; Figure 4.5), which was isolated from the high and

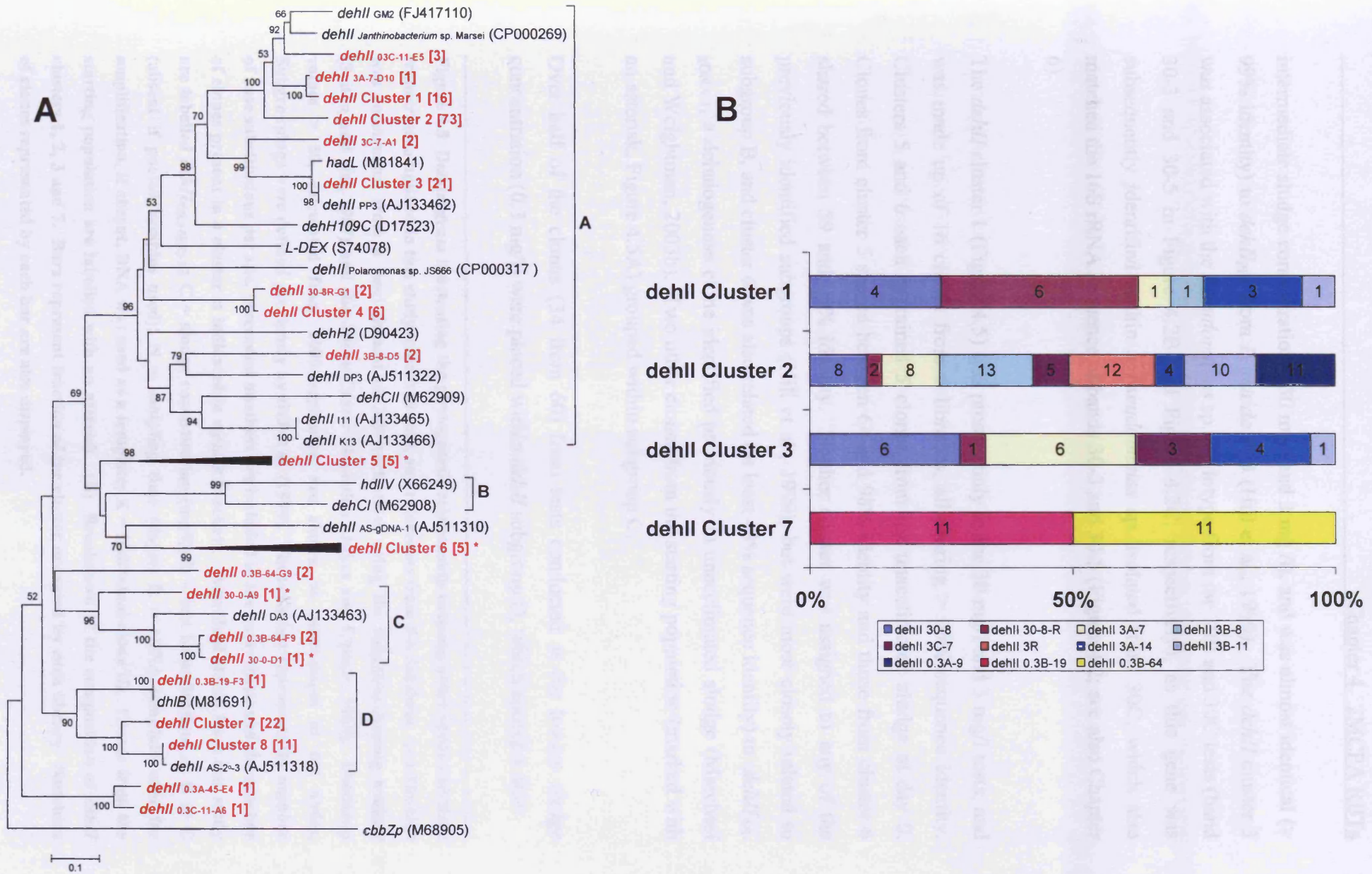

Figure 4.5 For legend, see next page.

intermediate sludge concentrations (30 mg/l and 3 mg /l), and was almost identical (> 99% identity) to *dehII*$_{PP3}$ from *P. putida* PP3 (Hill et al., 1999). The *dehII* cluster 3 was associated with the *Pseudomonas* sp. phylotype from the 30B and 30C tests (band 30-3 and 30-5 in Figure 4.2B and Figure 4.2C, respectively), as this gene was subsequently identified within a *Pseudomonas* sp. isolated from 30C, which also matched the 16S rRNA sequence of bands 30-3 and 30-5 (Figure 4.2; see also Chapter 6).

The *dehII* cluster 1 (Figure 4.5) was present only in the 30 mg/l and 3 mg/l tests, and was made up of 16 clones from 6 libraries, all sharing > 99% sequence identity. Clusters 5 and 6 each contained 5 clones from the unacclimated sludge at day 0. Clones from cluster 5 shared between 64 and 98% identity and those from cluster 6 shared between 59 and 99% identity. Neither cluster was assigned to any of the previously identified subgroups (Hill et al., 1999), but were most closely related to subgroup B, and cluster 6 was also related (at least 48% sequence identity) to *dehII*$_{AS\text{-}gDNA\text{-}1}$, a dehalogenase clone identified previously in unacclimated sludge (Marchesi and Weightman, 2003b). Two other clones from the starting population (marked with an asterisk, Figure 4.5A) grouped within subgroup C.

Over half of the clones (34 from 60) from tests conducted at the lowest sludge concentration (0.3 mg/l) were placed within *dehII* subgroup D, which contains *dhlB*

Figure 4.5 Dendrogram illustrating the phylogenetic relationship between *dehII* genes and their transcripts amplified in this study (coloured red), and references from the database. (A) The tree was constructed from a final dataset of 389 nucleotides using the Neighbor-Joining method (Saitou and Nei, 1987) and the Jukes-Cantor algorithm (Jukes and Cantor, 1969). Bootstrap values > 50, calculated from 1000 replicates, are shown as percentages at the nodes. Subgroupings were defined previously by Hill et al. (1999). The scale bar represents the number of base substitutions per site. Accession numbers are included in the parenthesis and the number of clones present in a cluster is indicated in square brackets. Individual clones from this study are labelled *dehII*$_{C(L)\text{-}N(R)\text{-}X}$; C = sludge concentration (mg/l); L = test identification; A, B or C (absent if pooled samples used); N = sampling time (days); R = cDNA template used for amplification, if absent, DNA was used as a template; X = Individual clone ID. Clones from the starting population are labelled with an asterisk. (B) Breakdown of the composition of *dehII* clusters 1, 2, 3 and 7. Bars represent fraction of the cluster occupied by each library. Numbers of clones represented by each bar are also displayed.

from *Xanthobacter autotrophicus* GJ10 (Janssen et al., 1985). Cluster 7 was made up of 22 clones sharing > 99% nucleotide sequence identity sampled from both phases of the 0.3B test (days 19 and 64), whilst cluster 8 contained only clones (> 99% nucleotide sequence identity) from the latter half of test 0.3A (day 45). Both clusters 7 and 8, though quite distinct (85% sequence identity), appeared to originate from the same *Starkeya* like phylotype, represented by DGGE bands 0.3-2/3 and 0.3-4/5 (Figure 4.2G and H, respectively).

The majority of clones from day 11 of test 0.3C were assigned to cluster 4 in *dehII* subgroup A and were closely related to two identical clones from the 30 mg/l tests ($dehII_{30\text{-}8\text{-}R\text{-}G1}$, Figure 4.5A). Another group of three identical clones from day 11 of the 0.3C test ($dehII_{0.3C11\text{-}E5}$, Figure 4.5A) were also assigned to subgroup A, but were quite distinct from those in cluster 4 (62% nucleotide identity). All other clones were assigned as individuals or pairs to subgroups A and C, with the exception of $dehII_{0.3A\text{-}45\text{-}E4}$ and $dehII_{0.3C\text{-}11\text{-}A6}$, which were present on a highly supported node outside of all previously characterised subgroups.

4.3 Discussion

Decreasing inoculum concentration affected the dechlorination kinetics of 2MCPA in two ways. The time taken to achieve total dechlorination increased with decreasing inoculum concentration, taking on average 8(±0), 11(±3.6) and 73(±31) days at 30, 3 and 0.3 mg/l, respectively (Figure 4.1). The dechlorination kinetics of each replicate test also became more variable as the inoculum concentration decreased, as indicated by the higher standard deviations of the mean dechlorination times (Figure 4.1). However, most of this variability was not associated with the initial onset of dechlorination, which commenced following a lag of between 3 and 8 days in all but the 0.3B tests, but with the re-commencement of dechlorination following a secondary lag phase. Having achieved 50% dechlorination, 5 of the 9 tests underwent a secondary lag phase lasting between 1 and 34 days, with the longest lags in the 0.3 mg/l tests. Other studies have also shown increasing lag phases with decreasing inoculum size (Ingerslev et al., 2000). A reduction in inoculum concentration is likely to reduce the number of specific degraders present within the system, which would in turn increase the time taken to form a strong degrading population, delaying the onset of degradation (Spain et al., 1980). Though this does not appear to be the case during the early stages, or first phase, of any of the test systems, it may certainly apply to the second phase of dechlorination.

DGGE analysis of the population change occurring during 2MCPA dechlorination may offer some clues as to the variability of function observed within the test systems. All phylotypes identified in this study were members of the *Proteobacteria* (Table 4.1), which is not a novelty in itself as the vast majority of αHA dehalogenating organisms identified to date are *Proteobacteria* (Marchesi and Weightman, 2003b). However, whilst tests having single phase dechlorination curves (30A-C and 3C, Figure 4.1) showed the enrichment of a defined population which was maintained throughout active dechlorination (Figure 4.2A-C and Figure 4.2F), biphasic curves, as seen in all other test systems, were associated with an altering of the population following 50% dechlorination (Figure 4.2), and was most evident in test 0.3A (Figure 4.2G). This suggests that the population change was related to the observed change in dechlorination kinetics (as seen in Chapter 3). A change of functionality can often be attributed to changes in the bacterial community, as

demonstrated during degradation of trichloroethene (Ayala-del-Río et al., 2004), crude oil (Macnaughton et al., 1999) and 2,4-dichlorophenol (Zhang and Wiegel, 1990). However, as demonstrated by Fernández et al. (1999), stability of function is not always reflective of a stable community profile. Using a glucose fed methanogenic reactor, they showed that a highly dynamic microbial community was able to stably maintain its ecosystem function, in terms of the chemical oxygen demand removal, over a 605 day period.

The change observed in the bacterial community structure following 50% dechlorination (Figure 4.2) was also mirrored, in three of the test systems, by a change in the dehalogenase gene pool; tests 3A, 0.3A and 0.3C (Figure 4.1B and C) clearly showed separate or staggered enrichment of the two dehalogenase genes, where *dehII* was enriched first, followed by *dehI* (Figure 4.3B and C).

Several factors may have caused or contributed to the succession of the 2MCPA degrading population initially formed in these tests. The formation of toxic intermediates of metabolism has been suggested as a mode of inhibiting xenobiotic degradation (McCarthy et al., 1997), although this is unlikely in this case as the product of 2MCPA dechlorination is pyruvate, which is a component of central metabolism and not inherently toxic to microbial cells. Protozoal predation, which has been shown previously to reduce bacterial numbers even during xenobiotic degradation (Cox et al., 1999; Thirup et al., 2000), may have reduced the numbers of the initially selected degrader sufficiently to allow another organism to out-compete it within the acclimated community. However, although protozoal predation was not measured in this analysis, it seems unlikely that this was the cause of the population shifts, at least for 3A and 3B, since there did not appear to be any significant change in DGGE band intensity for the *Herbaspirillum* (Figure 4.2D and E). Diauxic growth does not appear to be a likely cause of the secondary lag phase seen at lower sludge concentrations, as the availability of additional readily degradable carbon sources, added along with the inoculum, would be reduced considerably at lower inoculum concentrations. In addition, classical diauxic growth is associated with two separate growth phases for a single organism, whereas in several of the tests under investigation, the second dechlorination phase was associated with the enrichment of new organisms.

The partitioning of substrate dechlorination into two distinct phases, mirrored by a change in the bacterial community and dehalogenase gene pool, suggests a link to the racemic nature of 2MCPA, and the substrate specificity of the two αHA dehalogenase enzymes. DehI enzymes commonly utilise both D- and L-2MCPA isomers (Weightman et al., 1982; Liu et al., 1994), or in some cases, the D- isomer only (Leigh et al., 1988; Smith et al., 1990), whilst DehII enzymes are only known to act on the L- isomer (Klages et al., 1983; Tsang et al., 1988; Liu et al., 1994). Given the stereospecificity of the two dehalogenase enzymes outlined above, this suggests that L-2MCPA was degraded first by a DehII, leaving only D-2MCPA within the test system, creating a strong selection for a DehI containing organism, forcing the observed change to the microbial community (Figure 4.2D, G and I). Such a staggered enrichment of *dehI* and *dehII* was also observed in Chapter 3, and was putatively linked to a reduction in the abundance of *dehI* containing organisms within lyophilised activated sludge, relative to fresh activated sludge, and the degradation of L-2MCPA prior to D-2MCPA. Other studies have inferred a specific degradation mechanisms, based on the presence of certain catabolic genes at different stages of degradation, e.g. the degradation of shorter chain alkanes by group I alkane hydroxylases, prior to longer chain alkanes, which were subsequently degraded by group II alkane hydroxylases (Sei et al., 2003), or the association of catechol 2,3-dioxygenases with a possible *meta*-cleavage pathway for nonylphenol ethoxylates and nonyl phenol (Zhang et al., 2008).

Given the ability of the majority of previously described DehI enzymes to degrade D- and L-2MCPA (subgroups A and B, Figure 4.4A), it is perhaps surprising that *dehII* genes were commonly enriched prior to *dehI*s in those tests showing bi-phasic dechlorination curves, as this necessitates the subsequent enrichment of a *dehI* to completely dechlorinate 2MCPA. This is most likely a reflection of the environmental abundance of the two gene families, as *dehII* has been shown to be considerably more diverse and widely distributed than *dehI* (see Chapter 3; and Marchesi and Weightman, 2003). This was confirmed in the present study; for example in the 30 mg/l tests where *dehII* was detectable at time 0, but *dehI* was only detected following the commencement of dehalogenation (Figure 4.3). Reducing the inoculum concentration has been shown to preferentially affect those taxa present at

low abundance (Franklin and Mills, 2006; Wertz et al., 2007). As such, microbial functions carried out by smaller microbial populations, such as nitrification and methanogenesis, are more sensitive to changes in population diversity than broader functions such as decomposition (Toyota et al., 1999; Wu et al., 2002). It therefore appears likely that the correlation between lower sludge concentrations and bi-phasic dechlorination curves was due to a further reduction in size of an already small *dehI* containing community. It therefore follows that the secondary lag phase may represent the time taken to enrich a *dehI* containing organism from a very small starting population.

Community stability is related not only to the diversity of species present, but also to its functional redundancy (Wittebolle et al., 2009). For an organism to be redundant, it could be removed from the population without altering the functionality of that population (Gitay et al., 1996). The *dehII* gene pool present during testing was more diverse than the *dehI* gene pool. Thus, *dehII* clones grouped not only within the dominant clusters enriched during substrate dechlorination (as was the case for *dehI*, Figure 4.4), but were spread throughout each of the previously described subgroupings (Figure 4.5). There was also considerable diversity within the *dehII* gene pool in unacclimated sludge (clones marked with an asterisk in Figure 4.5), whereas *dehI* was below the level of detection. Such a diverse *dehII* gene pool suggests that the inoculum carries a strong functional redundancy for *dehII*, making it better able to resist a reduction in size, as shown previously in bacterial communities degrading simple compounds such as glucose and acetate (Franklin and Mills, 2006). This may explain the relatively consistent lag phase observed at the beginning of each test, regardless of inoculum concentration; i.e. even if an organism is lost due to reduced inoculum size, at least one organism is left capable of performing the same function. This was apparently not the case for *dehI*, which was not detectable until the onset of dechlorination in any of the tests, and not at any stage of test 0.3C, implying low functional redundancy within this population.

Test 0.3C apparently represents the total loss of a catabolic function, as no bacterial 16S rRNA or dehalogenase PCR products could be detected by day 42, despite the early appearance of a *Janthinobacterium*-like phylotype (Figure 4.2) and *dehII* PCR products (Figure 4.3) during days 9-18. This may be due to the complete loss of *dehI*

from the test system following a 100-fold reduction in inoculum concentration, though dechlorination did continue above the level observed in the abiotic control. It is possible that both the 16S rRNA and *dehI* gene primer sets used in this study could not amplify any bacteria present during the second half of dechlorination. However, it may also be the case that an archaeal or eukaryotic organism, which have been known to degrade αHA (Jensen, 1957b; Rye et al., 2009) was responsible for the dechlorination observed, as they would not have been detected by the 16S rRNA gene primers used in this study.

At the lower inoculum concentration (0.3 mg/l activated sludge) each replicate test system contains bacterial phylotypes and dehalogenases distinct from those present at higher inoculum concentrations (Figure 4.2; Table 4.1; Figure 4.4; Figure 4.5). This may be due to the removal of faster growing, more competitive organisms present at higher inoculum concentrations, allowing the enrichment of less competitive phylotypes containing novel dehalogenases. Therefore, the use of lower inoculum concentrations for bacterial enrichments may be of use in the identification of novel dehalogenating bacteria in future studies, as shown previously for the identification of novel marine bacteria (Button et al., 1993; Toffin et al., 2004; Giovannoni et al., 2007).

Interestingly, even though an identical *Starkeya*-like phylotype was present in tests 0.3A and 0.3B (Figure 4.2G and H, respectively), they did not appear to share the same dehalogenases. The *Starkeya* from test 3A (Figure 4.2G) was associated with *dehI* cluster 4 (Figure 4.4A) and *dehII* cluster 8 (Figure 4.5A), whereas the *Starkeya* from test 0.3B (Figure 4.2H) was associated with *dehI* cluster 3 (Figure 4.4A) and *dehII* cluster 7 (Figure 4.5A). An alignment of several *Starkeya* (formerly *Thiobacillus novellus*; Kelly et al., 2000) 16S rRNA gene sequences showed that variability within these sequences existed outside of the V3 variable region, which was the region amplified in this study (results not shown). It is therefore conceivable that despite sharing identical 16S rRNA gene sequences for the V3 region, that the two *Starkeya* phylotypes from 0.3A and 0.3B are actually different organisms. Other studies have identified organisms with identical 16S rRNA gene sequences, which

were actually genetically and functionally distinct from each other (Jaspers and Overmann, 2004).

It is also possible that the same organism present in the unacclimated sludge of both test systems (0.3A and 0.3B; Figure 4.2G and H, respectively), acquired different dehalogenases via horizontal gene transfer (HGT). Dehalogenase genes are commonly associated with mobile genetic elements such as plasmids and insertion sequences (Kawasaki et al., 1984; Kawasaki et al., 1994; Sota et al., 2002; Weightman et al., 2002) and the horizontal distribution of plasmid borne αHA dehalogenases has been shown previously (Hill and Weightman, 2003).

One of the tests, 0.3B, apparently contained the same organism (Figure 4.2H), harbouring both a *dehI* and *dehII* gene (Figure 4.3C, Figure 4.4A, Figure 4.5A), for the duration of the analysis, and yet still gave a two-phase dechlorination curve. This is surprising, as one would expect such an organism to grow on 2MCPA, unhindered by its racemic nature. It is possible that the presence of one isomer actively inhibits the degradation/uptake of the other in this organism representing some form of diauxic growth, however this explanation does not account for the long lag phase (~ 15 days) between the two dechlorination phases, as enzyme induction/de-repression would be unlikely to take so long. Another possible explanation is that one of the dehalogenases, though present at the beginning of the experiment, was a silent gene not giving a functional dehalogenase enzyme which, on depletion of one of the two 2MCPA isomers, was decryptified to avoid starvation of the cells. Decryptification or activation of silent genes in response to environmental stresses such as starvation has been reported previously (Matin et al., 1989). The activation of a silent dehalogenase gene could also account for the length of the secondary lag phase in test 0.3B. There are various mechanisms where by silent genes become activated (decryptified), e.g. deletion of genetic material, which is responsible for activating a silent glutamate dehydrogenase gene (*gudB*) in *Bacillus subtillis* (Belitsky and Sonenshein, 1998; Commichau et al., 2008), or activity of insertion sequences (IS) such as IS*1* and IS*5*, which activate the *bgl* operon in *Escherichia coli* K12 (Reynolds et al., 1981). There is also evidence to suggest that IS are more active when cells undergo periods of stress such as carbon starvation (Hall, 1999b), thus increasing the probability of

activating silent degradative pathways exactly when they are needed (Hall, 1999a). Silent αHA dehalogenase genes, both *dehI* and *dehII*, have been reported previously (Hill et al., 1999; Tsang and Sam, 1999). Decryptification of dehalogenase genes, and chloroamidase genes from *Pseudomonas* spp. in response to a number of environmental stresses has also been shown previously (Hope and Slater, 1995).

At inoculum concentrations below 30 mg/l, considerable variability was observed in RBT pass/fail outcome. A 10-fold reduction of inoculum concentration was almost sufficient to cause test 3A to fail, whilst a 100 fold reduction caused all replicates to fail. Such variability was probably due to reduced functional redundancy within the community, i.e. the loss, or reduction in number of certain specific degraders from the starting inoculum, as indicated by the delayed enrichment of *dehI* relative to *dehII* in several of the tests conducted at lower sludge concentrations, and the complete absence of *dehI* from test 0.3C. This effect may be mimicked by the addition of a more recalcitrant chemical to the test system, as a given inoculum would likely contain fewer specific degraders capable of its biodegradation, and as such would carry a reduced functional redundancy, which has been shown in this study to increase variability within RBTs.

Using molecular genetic analysis of the microbial population and in particular, the catabolic gene pool, this study suggests that a lack of specific degraders within the system, associated with the loss of functional capability of the population in the form of *dehI*, was responsible for the failure of 2MCPA using a model RBT protocol. Given the rigidly placed maximum inoculum concentration necessitated by the non-specific detection methods used to monitor degradation in the RBTs, it may be advisable to allow some pre-adaptation, i.e. pre-exposure of an inoculum to the test compound, prior to testing in order to maximise the probability of biodegradation (Thouand et al., 1996).

5. The Effects of Varying Inoculum Concentration on Biodegradation of Trichloroacetic Acid (TCA) In a Model Ready Biodegradation Test (RBT) System

5.1 Introduction

Trichloroacetic acid (TCA) has been used extensively in the past as a broad spectrum herbicide for the control of monocotyledons, principally wild grasses, though its use has since been banned in several European countries due to concerns over its human and animal toxicity (Juuti and Hoekstra, 1998), and the introduction of more effective herbicides (McCulloch, 2002). There has been some speculation that TCA is responsible for a significant part of forest die-back, though evidence supporting these claims is largely circumstantial (Sutinen et al., 1995; McCulloch, 2002).

There are now known to be multiple sources of environmental TCA, as traditional views that TCA was formed exclusively as a result of human activity have been challenged recently. Haloacetic acids, especially TCA, are readily produced from fulvic acids as a result of water purification by chlorination (McCulloch, 2002) and from pulp bleaching during paper production (Juuti et al., 1995; Dalvi et al., 2000). TCA concentrations as high as 7,600 μg/l were detected in untreated bleaching plant effluents (Yu and Welander, 1994), though more typical levels would be 100 μg/l for industrial treated wastewater and 1-10 μg/l in treated domestic waste water and drinking water (EuroChlor, 2001). Due to its high solubility, TCA produced during water purification and paper production would escape directly into the environment and accumulate in the hydrosphere; including soil water, ground water, lake and river waters (Laturnus et al., 2005). TCA is also formed as a result of atmospheric oxidation of the chlorinated hydrocarbon solvents tetrachloroethene and 1,1,1-trichloroethane of anothropogenic origins (Matucha et al., 2003). As mentioned above, it is well known that chloroacetic acids, including TCA, are readily produced by chlorination of drinking water. Haiber et al. (1996) demonstrated TCA production when humic acids and a range of short chain aliphatic acids were incubated in the presence of a chloroperoxidase (CPO) from the fungus *Caldarwmyces fumago*, sodium chloride, and hydrogen peroxide. Given that TCA is formed via a CPO-mediated reaction, and CPO-like activity has been detected in soils, it is reasonable to assume that TCA formation may occur naturally in soils (Hoekstra et al., 1999), as

has since been shown in forest soil microcosms via both biotic, and putatively abiotic processes (Matucha et al., 2007). TCA is detectable in some old ice and firn (old compacted snow) samples dating back to the early 19th century, long before industrial production of reactive chlorine, which implies a background level of naturally produced TCA (Haiber et al., 1996; McCulloch, 2002).

The mechanism of TCA dechlorination is unclear, though it is known to be degraded both aerobically and anaerobically. Early reports described a taxonomically unidentified bacterium, several *Arthrobacter* spp. and a *Pseudomonas* sp. capable of degrading TCA in the presence of additional co-factors and carbon sources (Jensen, 1957a, 1960; Kearney et al., 1969). Indeed, it is characteristic of TCA degraders that most are unable to utilise TCA as the sole source of carbon and energy, possibly accounting for the lack of well characterised TCA degraders in the literature (Weightman et al., 1992). More recently, a *Gammaproteobacterium*, closely related to *Acinetobacter calcoaceticus*, was isolated capable of growth on up to 20 mM TCA as the sole source of carbon and energy, but was unable to grow on monochloroacetic acid (MCA) or dichloroacetic acid (DCA) (Yu and Welander, 1995). Oxalic acid has been reported as the initial product of aerobic TCA degradation (Motosugi et al., 1982; Ellis et al., 2001), though other pathways may also exist. Weightman et al. (1992) were unable to detect oxalate following TCA degradation and speculated that dehalogenation may involve the formation of carbon monoxide (CO) and CO_2. Stringfellow et al. (1997) also described the formation of CO following an initial dehalogenation of TCA. Reductive dehalogenation of TCA has also been described for *Trichlorobacter thiogenes* gen nov., sp. nov., and appears to act via a sulphur-sulfide redox cycle, achieving TCA reduction using electrons derived from acetate oxidation (de Wever et al., 2000). Anaerobic degradation of TCA and other chlorinated organic compounds has also been described (Yu and Welander, 1994).

As shown in Chapter 4, varying the inoculum size during RBTs can have a profound effect on test outcome, driven in part by the diversity and functional redundancy of the inoculum. Under standard RBT conditions, 2-monochloropropionic acid (2MCPA; the test substrate used in Chapter 4) was readily degraded by the activated sludge inoculum. In preliminary experiments, TCA was shown to be more recalcitrant than 2MCPA, and has been shown previously to be more recalcitrant than

either MCA or DCA (Ellis et al., 2001). As such, this study aimed to investigate the effect of increasing inoculum size on the RBT outcome for TCA, which was shown in preliminary work to fail under standard RBT conditions. Cultivation-independent methods were used to determine changes in the bacterial community and dehalogenase genes during the enrichment process.

5.2 RESULTS

5.2.1 TCA dechlorination at four different sludge concentrations

Four different activated sludge concentrations; 30, 60, 120 and 300 mg/l, were tested in replicates for their ability to degrade TCA at a concentration of 20 mgC/l using a modified RBT protocol. Taking average values from replicate experiments, total dechlorination of TCA was achieved in 30, 32, 35.5 and 48 days for the 300, 120, 60 and 30 mg/l tests respectively (Figure 5.1). A significant lag phase (estimated as the time taken to achieve 10% dechlorination) was observed at all inoculum concentrations tested. A lag of 24 days was observed in the 300 mg/l tests, with lags of 26, 28 and 30 days for tests conducted at 120, 60 and 30 mg/l sludge, respectively. At the highest sludge concentration (300 mg/l; 10-fold higher than the standard inoculum for RBTs) TCA only just reached the 70% pass level within the 28 day period required by the RBT, but it failed the test at all other sludge concentrations.

5.2.2 Community analysis by 16S rRNA gene DGGE

Selected time points from each test were chosen to study the changing bacterial population during the course of TCA degradation, using DGGE of the 16S rRNA gene. DGGE profiles were very similar for all tests studied (Figure 5.2) and highlighted two distinct population changes. The appearance of a *Dokdonella*-like phylotype (*Gammaproteobacteria*) during the lag phase (bands marked A, Figure 5.2; Table 5.1), and the appearance of a *Bradyrhizobium* like phylotype (*Alphaproteobacteria*) following the onset of dechlorination (bands marked B, Figure 5.2; Table 5.1) was common to all tests. Both 30 mg/l RBTs showed the enrichment of another phylotype (band 30-D, Figure 5.2; Table 5.1) closely related to a *Methylibium* sp. (*Betaproteobacteria*), around day 44, not identifiable in any other test during active TCA dechlorination. A phylotype affiliated to the *Bacteroidetes* appeared at a single time point, day 29, of the 120_2 test (120-C, Figure 5.2C; Table 5.1). This phylotype was not seen in the other 120 mg/l test, nor was it detected during any of the other tests.

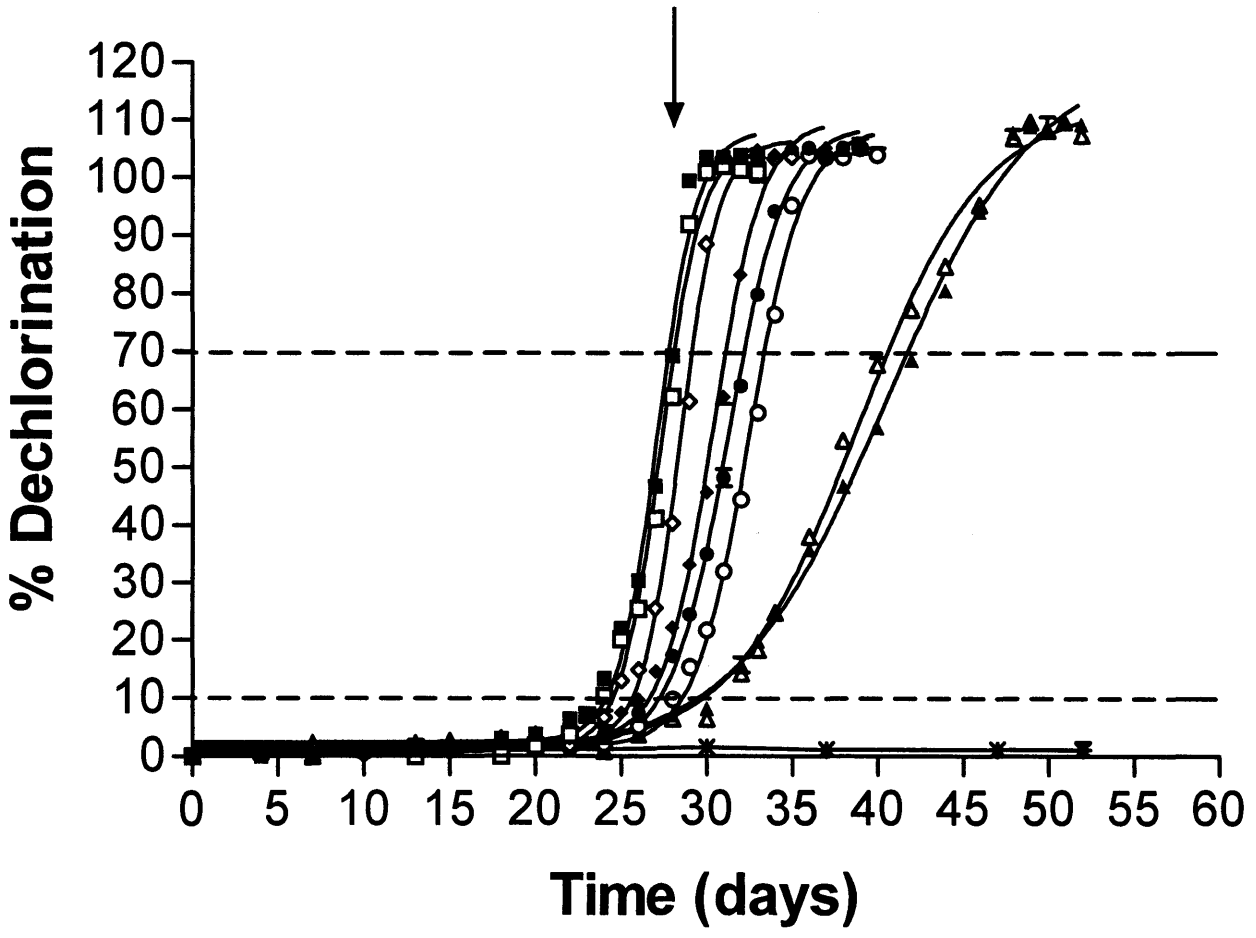

Figure 5.1 TCA dechlorination at different activated sludge concentrations. ■ and □ represent 300 mg/l sludge replicates 300-1 and 300-2, respectively. ◆ and ◇ represent 120 mg/l sludge replicates 120-1 and 120-2 respectively. ● and ○ represent 60 mg/l sludge replicates 60-1 and 60-2, respectively. ▲ and △ represent 30 mg/l sludge replicates 30-1 and 30-2, respectively. ✳ represents the abiotic control. Broken horizontal lines represent biodegradation start point (10%) and pass level (70%). Arrow indicates the 28 day cut-off for a RBT pass. Error bars represent the standard error of the mean (n = 3).

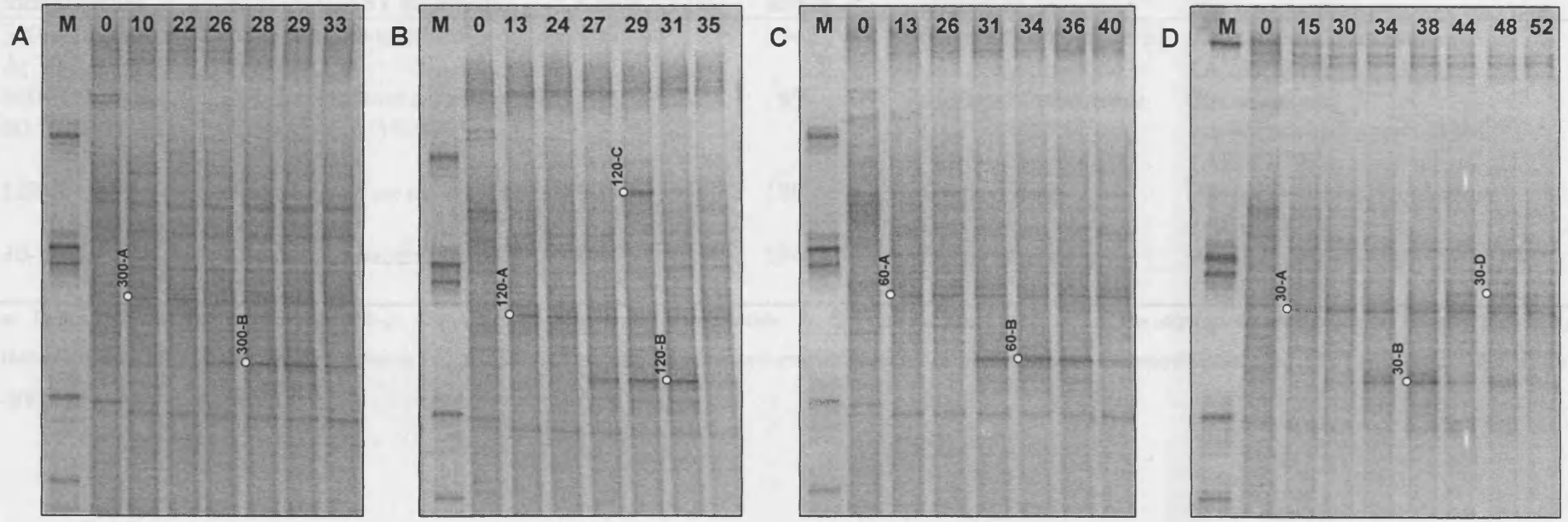

Figure 5.2 DGGE of the 16S rRNA genes from selected time points during one of two replicate TCA RBTs conducted at different activated sludge concentrations. Replicate tests conducted with the same inoculum concentration gave very similar DGGE profiles (data not shown). (A) 300-2 test (300 mg/l activated sludge). (B) 120-2 test (120 mg/l activated sludge). (C) 60-2 test (60mg/l activated sludge). (D) 30-2 test (30 mg/l activated sludge). Numbers above each lane represent time (days) of sampling, M = marker. Open circles indicate bands excised for DNA sequencing (see Table 5.1).

Table 5.1 Closest sequence matches to excised DGGE bands using the MEGABLAST algorithm and the ribosome database project (RDP).

Band identification	Top matches using MEGABLAST algorhithm	Sequence similarity (%)	Sequence length	Phylogenetic affiliation	Top matches using RDP
300-A; 120-A; 60-A; 30-A[a]	Uncultured bacterium (FM174358)	100	194	*Gammaproteobacteria*	*Dokdonella koreensis* (AY987368)
300-B; 120-B; 60-B; 30-B	*Bradyrhizobium elkanii* strain CCBAU (GU552899)	100	169	*Alphaproteobacteria*	*Pseudomonas carboxydohydrogena* DSM[b, c] (AB021393)
120-C	*Flavobacterium* sp. A6ATF1 (FN293287)	99	189	*Bacteroidetes*	*Flavobacterium frigidarium* (AF162266)
30-D	Uncultured bacterium (GQ500805)	98	194	*Betaproteobacteria*	*Methylibium petroleiphilum* PM1 (AF176594)

a. Band 30-A differed from bands 300-A, 120-A and 60-A by a single nucleotide. b. Several different members of the *Alphaproteobacteria* gave equally high hits, including members of the *Bradyrhizobium*, *Nitrobacter*, *Afipia* and *Rhodopseudomonas* genera. c. *Pseudomonas carboxydohydrogena* DSM was reclassified as an *Afipia* sp. (Anzai et al., 2000).

5.2.3 Analysis of dehalogenase genes and their transcripts by (RT)-PCR

Selected time points from each test system were assayed by PCR using the degenerate primers of Hill et al. (1999), for the presence of *dehI*, *dehII*, and their transcripts. Figure 5.3 typifies the results seen in all tests, irrespective of sludge concentration. Both *dehI* PCR primer sets ($dehI_{F1\text{-}R1}$, $dehI_{F1\text{-}R2}$) were used to screen test samples for the presence of *dehI*. None of the samples tested positive for the larger of the two *dehI* PCR products. The shorter *dehI* PCR products were not detectable in any of the tests at day 0, and remained undetectable during the lag phase, until or just prior to the onset of dechlorination at days 22, 24, 26 and 30 for the 300, 120, 60 and 30 mg/l tests, respectively (Figure 5.3A). All time points sampled thereafter gave positive *dehI* PCR products. The *dehI* transcripts were also detected in all test systems, though only during substrate dechlorination, at days 26, 28 and 29 for test 300-1 (Figure 5.3B) and 300-2; days 24, 28 and 31 (120-1); 24, 27 and 29 (120-2); 26, 30 and 33 (60-1); 31, 34 (60-2); 34, 38, 44 and 48 (30-1 and 30-2).

dehII PCR products of the expected size were amplified from every time point studied (Figure 5.3), but *dehII* transcripts were not detected in any of the enrichments, except for the 30 mg/l test (30-1 and 30-2), where very weak RT-PCR products were detectable during days 34, 38, 44 and 48 (Figure 5.4A). These RT-PCR products were shown not to be the result of DNA contamination (Figure 5.4B).

5.2.4 Diversity within the dehalogenase gene pool

Due to the enrichment of identical organisms in all RBTs analysed, dehalogenase clone libraries were constructed from samples taken during the lag phase, and during substrate dechlorination for the 300 and 30 mg/l tests only, to identify the variability present within the dehalogenase gene pool. Samples from replicate tests were pooled for clone library construction (see Table 5.2).

All 30 clones analysed from the *dehI* clone libraries were assigned to subgroup B (Figure 5.5). Within subgroup B, *dehI* cluster 1 contained 7 identical clones from the $dehI_{T300\text{-}29\text{-}D}$ and $dehI_{T30\text{-}44\text{-}D}$ libraries, and a single clone from the $dehI_{T300\text{-}29\text{-}R}$ library, differing from the others by a single nucleotide. A group of 6 identical clones from the $dehI_{T300\text{-}29\text{-}R}$ shared ≥ 98% nucleotide sequence identity with *dehI* cluster 1,

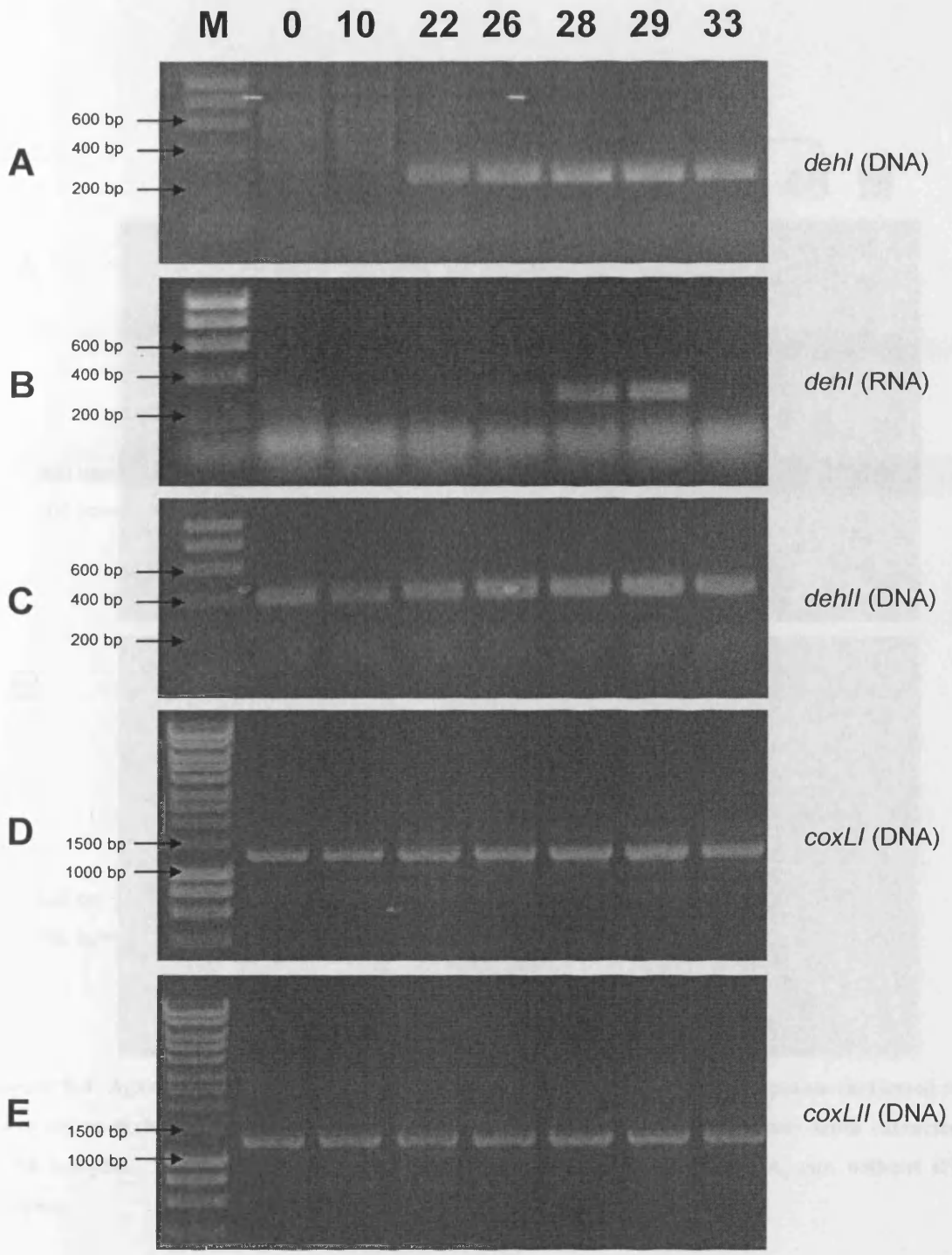

Figure 5.3 Agarose gels showing (RT)-PCR products from selected time points (indicated in days above each lane) for various functional genes from the 300-1 test: (A) *dehI*; (B) *dehI* (RT-PCR); (C) *dehII*; (D) Form I *coxL*; (E) Form II *coxL*. M = marker. This gel is representative of those obtained from all sludge concentrations tested.

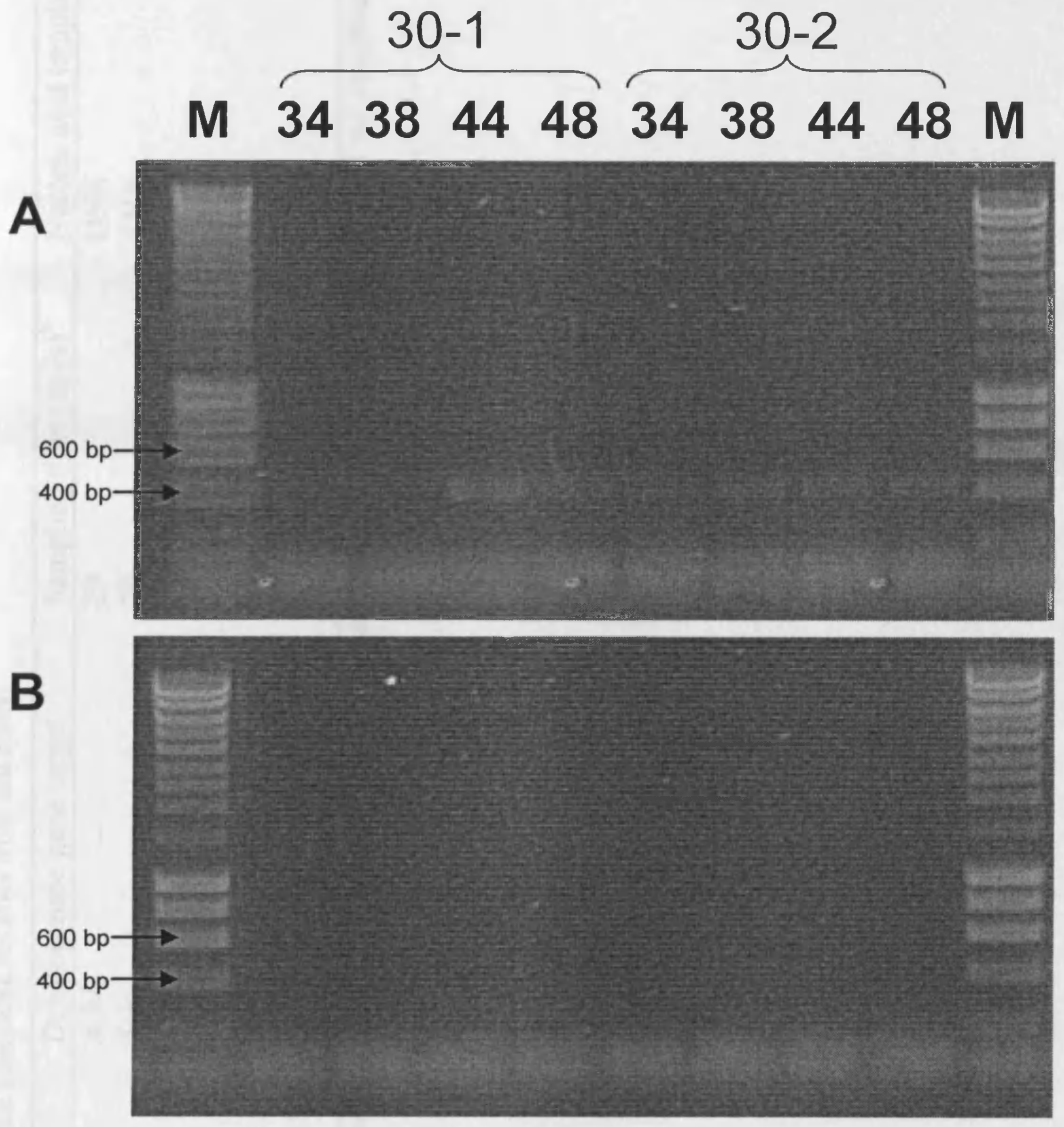

Figure 5.4 Agarose gel showing *dehII* RT-PCR products from selected time points (indicated in days above each lane) of the 30-1 and 30-2 tests. (A) *dehII* RT-PCR products from extracted RNA samples. (B) Negative control reactions for the same samples as in A, run without RT enzyme.

Table 5.2 Origin and identification of αHA dehalogenase gene clone libraries from this study.

Library name[a]	Test ID	Dehalogenase gene target	Sampling time (days)[b]	Nucleic acid template
$dehI_{T300\text{-}29\text{-}D}$	Pooled 300-1; 300-2	*dehI*	29	DNA
$dehI_{T300\text{-}29\text{-}R}$	Pooled 300-1; 300-2	*dehI*	29	RNA
$dehII_{T300\text{-}10\text{-}D}$	Pooled 300-1; 300-2	*dehII*	10	DNA
$dehII_{T300\text{-}29\text{-}D}$	Pooled 300-1; 300-2	*dehII*	29	DNA
$dehI_{T30\text{-}44\text{-}D}$	Pooled 30-1; 30-2	*dehI*	44	DNA
$dehII_{T30\text{-}15\text{-}D}$	Pooled 30-1; 30-2	*dehII*	15	DNA
$dehII_{T30\text{-}44\text{-}D}$	Pooled 30-1; 30-2	*dehII*	44	DNA

a. Library names are labelled $dehI/II_{TC\text{-}N\text{-}X}$; TC = sludge concentration (mg/l); N = sampling time (days); X = nucleic acid template used for amplification (D= DNA, R = RNA). b. Refer to Figure 5.1.

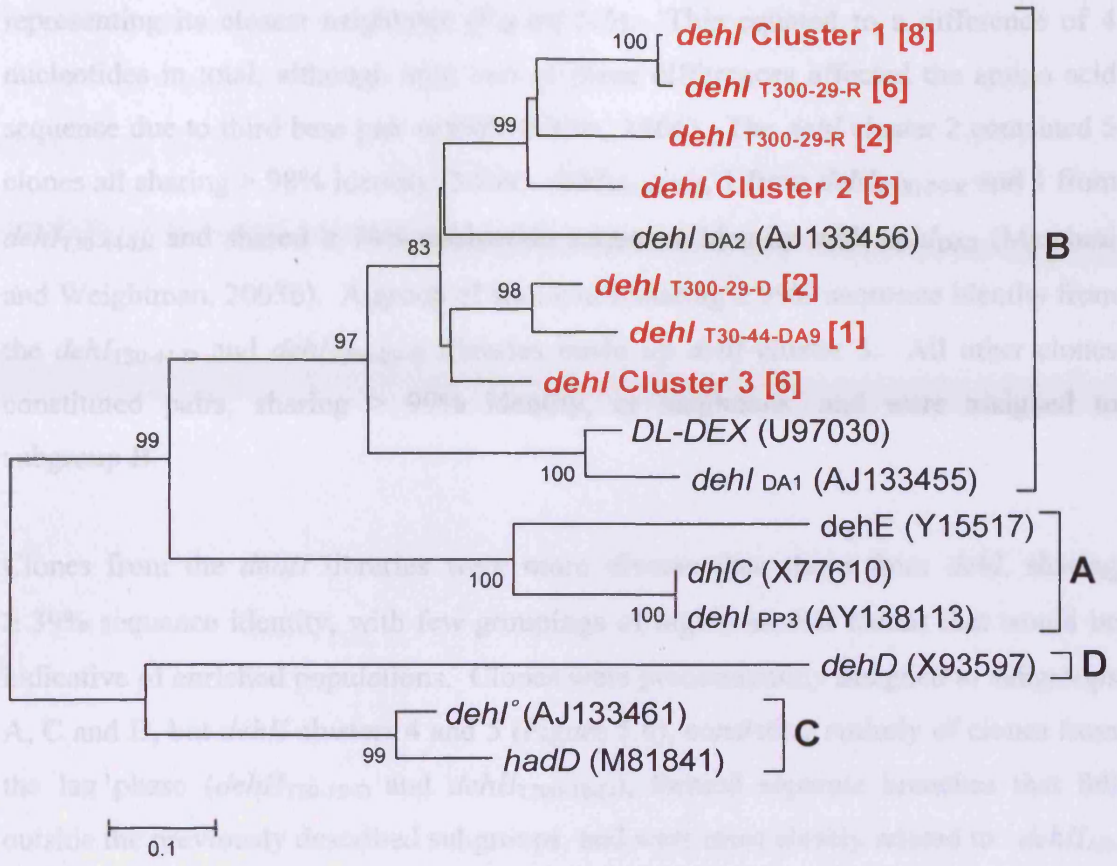

Figure 5.5 Dendrogram illustrating the phylogenetic relationship between *dehI* genes and their transcripts amplified from tests 300-1, 300-2, 30-1 and 30-2 (red), and references from the database. The tree was constructed from a final dataset of 222 nucleotides using the Neighbor-Joining method (Saitou and Nei, 1987) and the Jukes-Cantor algorithm (Jukes and Cantor, 1969). Bootstrap values > 50, calculated from 1000 replicates, are shown as percentages at the nodes. Subgroups were defined previously by Hill et al. (1999). The scale bar represents the number of base substitutions per site. Accession numbers are included in the parenthesis and the number of clones present in a cluster is indicated in square brackets. Individual clones from this study are labelled *dehI*$_{TC\text{-}N\text{-}XY}$; TC = sludge concentration (mg/l); N = sampling time (days; see Figure 5.1); X = nucleic acid template used for amplification (D= DNA, R = mRNA); Y = Individual clone ID. Clusters were grouped based on a nucleotide sequence identity of > 98%.

representing its closest neighbour (Figure 5.5). This equated to a difference of 4 nucleotides in total, although only two of these differences affected the amino acid sequence due to third base pair wobble (Crick, 1966). The *dehI* cluster 2 contained 5 clones all sharing > 98% identity; 3 from $dehI_{T300\text{-}29\text{-}D}$, 1 from $dehI_{T300\text{-}29\text{-}R}$ and 1 from $dehI_{T30\text{-}44\text{-}D}$, and shared ≥ 74% nucleotide sequence identity with $dehI_{DA2}$ (Marchesi and Weightman, 2003b). A group of six clones sharing ≥ 99% sequence identity from the $dehI_{T30\text{-}44\text{-}D}$ and $dehI_{T300\text{-}29\text{-}D}$ libraries made up *dehI* cluster 3. All other clones constituted pairs, sharing > 99% identity, or singletons, and were assigned to subgroup B.

Clones from the *dehII* libraries were more diverse than those from *dehI*, sharing ≥ 39% sequence identity, with few groupings of highly similar clones that would be indicative of enriched populations. Clones were predominantly assigned to subgroups A, C and D, but *dehII* clusters 4 and 5 (Figure 5.6), consisting entirely of clones from the lag phase ($dehII_{T30\text{-}15\text{-}D}$ and $dehII_{T300\text{-}10\text{-}D}$), formed separate branches that fell outside the previously described subgroups, and were most closely related to $dehII_{AS\text{-}gDNA\text{-}1}$ (Marchesi and Weightman, 2003b). A group of six clones from the TCA-acclimated sludge samples ($dehII_{T30\text{-}44\text{-}D}$ and $dehII_{T300\text{-}29\text{-}D}$) sharing > 99% sequence identity made up *dehII* cluster 1 within subgroup C (Figure 5.6). Another group of clones, sharing > 99% sequence identity, from the acclimated sludge samples ($dehII_{T30\text{-}44\text{-}D}$ and $dehII_{T30\text{-}29\text{-}D}$) formed *dehII* cluster 2 (Figure 5.6). The *dehII* cluster 3 was a more diverse group of 6 clones from the $dehII_{T300\text{-}29\text{-}D}$ and $dehII_{T30\text{-}44\text{-}D}$ libraries, all sharing ≥ 71% nucleotide sequence identity (Figure 5.6). Another diverse cluster of 3 clones, sharing 74-78% nucleotide sequence identity, from the lag phase of the 300 mg/l RBTs ($dehII_{T300\text{-}10\text{-}D}$ library) formed a clade between clusters 2 and 3 in subgroup C. A highly supported lineage represented by 7 identical clones from the lag phase of the 30 mg/l tests ($dehII_{T30\text{-}15\text{-}D}$) was most closely related to *hadL* (Barth et al., 1992), and represented the single largest group of identical clones within the *dehII* analysis. The remaining *dehII* clones were scattered throughout subgroups A, C and D as singletons or identical pairs.

5.2.5 Presence of carbon monoxide dehydrogenase (*coxL*) genes

Form I and form II *coxL* genes were detected in all RBTs and at all time points analysed (Figure 5.3D and E), but it was not possible to determine whether or not these genes were enriched during TCA dechlorination.

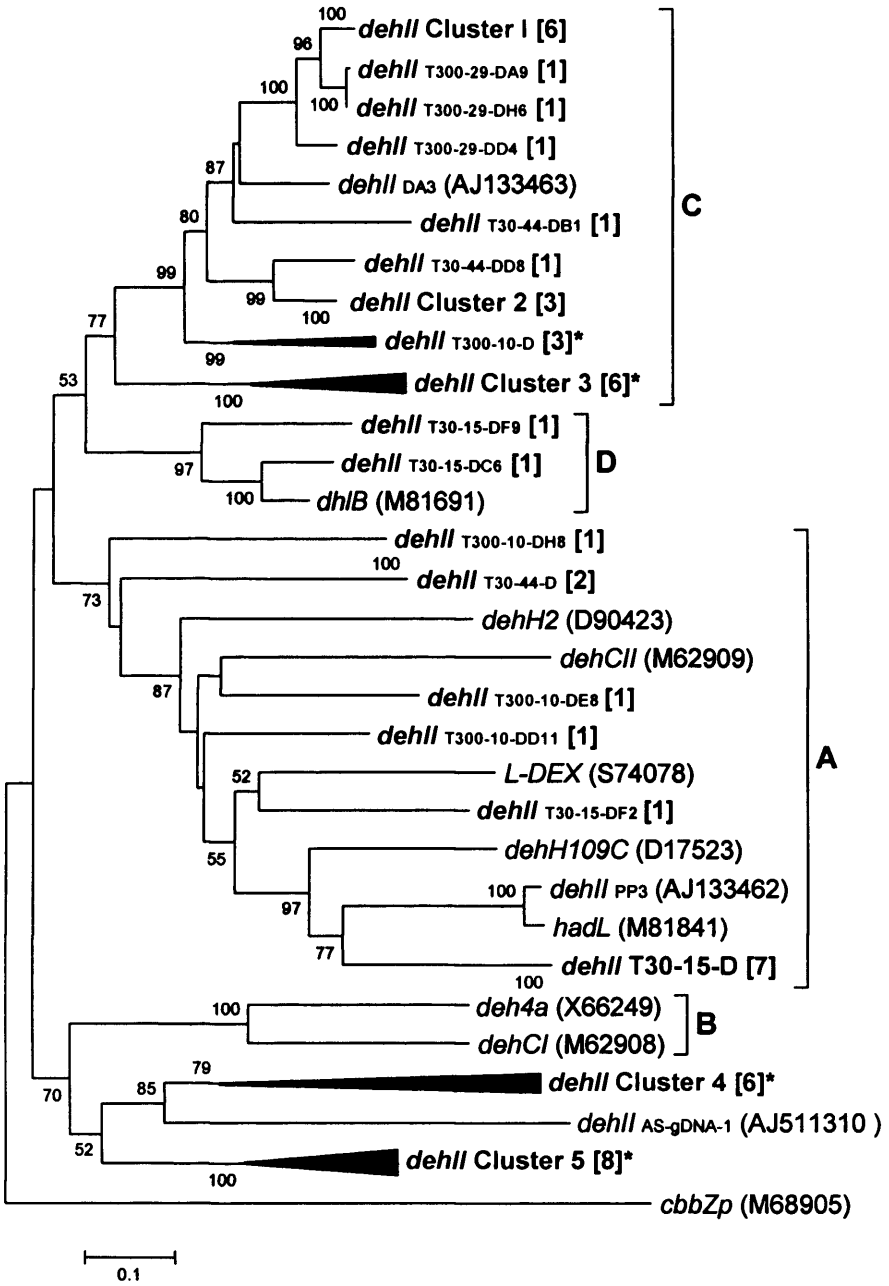

Figure 5.6 Dendrogram illustrating the phylogenetic relationship between *dehII* genes and their transcripts amplified from tests 300-1, 300-2, 30-1 and 30-2 (red), and references from the database. The tree was constructed from a final dataset of 353 nucleotides using the Neighbor-Joining method (Saitou and Nei, 1987) and the Jukes-Cantor algorithm (Jukes and Cantor, 1969). Bootstrap values > 50, calculated from 1000 replicates, are shown as percentages at the nodes. Subgroups were defined previously by Hill et al. (1999). The scale bar represents the number of base substitutions per site. Accession numbers are included in the parenthesis and the number of clones present in a cluster is indicated in square brackets. Individual clones from this study are labelled *dehII*$_{TC\text{-}N\text{-}XY}$; TC = sludge concentration (mg/l); N = sampling time (days; see Figure 5.1); X = nucleic acid template used for amplification (D= DNA, R = mRNA); Y = Individual clone ID. Clusters were grouped based on a nucleotide sequence identity of > 99%, except those marked with an asterisk which were grouped based on a nucleotide sequence identity of > 54%.

5.3 Discussion

All RBTs carried out in this study were associated with lengthy lag phases prior to the onset of TCA dechlorination, the duration of which was negatively correlated with inoculum concentration. At 300 mg/l activated sludge, 70% dechlorination of TCA was observed within the 28 day RBT period, but the length of the lag phase caused TCA to fail RBTs at all other concentrations tested. Lag phases of 10-77 days prior to detectable TCA dechlorination have been described previously, though much higher concentrations of TCA (5-30 mM) were used (Weightman 1992). Lag phases of very similar length to those reported in this study were also observed in preliminary studies of TCA degradation (results not shown). Other studies have shown that increasing inoculum size reduced the length of the lag phase prior to diethylene glycol (van Ginkel et al., 1995) or *p*-nitrophenol (Ingerslev et al., 2000) degradation.

In each test, the lag phase was associated with the appearance of an organism closely related to *Dokdonella koreensis* (Figure 5.2; Table 5.1) a *Gammaproteobacterium* originally isolated from soil on the island of Dokdo in the East Sea of Korea (Yoon et al., 2006) which has not previously been detected in activated sludge, or associated with degradation of halogenated compounds or other xenobiotics. This organism was not a dominant member of the unacclimated activated sludge inoculum in any of the RBTs, and its appearance did not coincide with the beginning of TCA dechlorination (Figures 5.1 and Figure 5.2) or the enrichment and expression of αHA dehalogenases, which were enriched only in association with TCA dechlorination (Figure 5.3). This organism was probably a highly competitive member of the starting inoculum which out-competed other community members for other available carbon sources. If this was the case, then it is possible that other endogenous carbon sources present in the inoculum were utilised in preference to TCA during the lag phase. Other studies have shown that the presence of additional readily degradable substrates can negatively impact upon a communities ability to degrade xenobiotics, such as *p*-nitrophenol (Swindoll et al., 1988) or 3-chlorobenzoate (Becker et al., 2006), where xenobiotic degradation was delayed until the levels of additional carbon were reduced. However, if this were the case, it would be expected that the length of the lag phase would be positively, not negatively, correlated to the inoculum concentration. It would therefore be of interest to monitor the degradation of endogenous carbon sources from

the inoculum during the lag phase (e.g. by CO_2 evolution or a reduction in dissolved organic carbon) in order to assess its affect on TCA degradation.

Given that the *Dokdonella* phylotype persisted within all RBTs even during TCA dechlorination (Figure 5.2), it is conceivable that it was somehow involved in TCA degradation. This could be a direct involvement, using a previously uncharacterised TCA degradation pathway, or an indirect involvement, by promoting and/or supporting the growth of the putative TCA degrader (*Bradyrhizobium* phylotype). Microbial consortia are commonly involved in xenobiotic degradation; for example, de Souza et al. (1998) described a two member bacterial consortium capable of degrading the chlorinatied *s*-triazine herbicide atrazine in sequential catabolic steps, and identified the genetic basis for this relationship. Other xenobiotic degrading consortia have also been identified, including a 3-methylhexanoic acid degrading consortium isolated from river sediment (Chua et al., 2001), and a 4-chloroaniline degrading consortium identified from Indonesian soils (Radianingtyas et al., 2003). The degradation of αHAs by microbial consortia has also been reported previously, where primary degradation (dechlorination) of the herbicide dalapon resulted in cross feeding of other community members with the metabolite, pyruvate (Senior et al., 1976).

It is reasonable to assume that reducing the inoculum concentration below 300 mg/l in these experiments would result in an initially smaller TCA degrading population, which may take longer to establish a stable population with detectable activity against TCA, as has been suggested previously during the degradation of various xenobiotics (Spain et al., 1980; Ventullo and Larson, 1986), including 2MCPA (see Chapter 4). Whilst this would not necessarily account for the length of the lag phase, it could explain the increased length of the lag phase as the inoculum concentration was reduced (Figure 5.1) and is supported by the increasingly delayed appearance of the *Bradyrhizobium* phylotype and *dehI* genes at lower inoculum concentrations.

In all RBTs in this study, TCA dechlorination was associated with the enrichment of a *Bradyrhizobium* like organism (Figure 5.2; Table 5.1) coincident with the enrichment, and expression of *dehI* genes (Figure 5.3A and B). Aerobic biodegradation of αHA is

almost invariably associated with two evolutionarily unrelated enzyme families, the group I (DehI) and group II (DehII) αHA dehalogenases (Hill et al., 1999). Group II αHA dehalogenases are more abundant and diverse in environmental samples, such as activated sludge, than the group I dehalogenases (see Chapters 3 and 4; Marchesi and Weightman, 2003a). This was also the case in this study, where *dehII* PCR products were detectable in unacclimated sludges, though *dehI* PCR products were not (Figure 5.3). The enrichment of *dehI* genes and the detection of *dehI* transcripts correlated with TCA dechlorination in all of the RBTs, regardless of inoculum concentration (Figure 5.1, Figure 5.3), suggesting that DehI, and not DehII activity was responsible for TCA dechlorination. *Bradyrhizobium* sp. containing both *dehI* and *dehII* genes have been identified previously from enrichments using αHA (Marchesi and Weightman, 2003a; Marchesi and Weightman, 2003b), but none have been reported with activity against TCA. All *dehI* genes identified in this study were most closely related to $dehI_{DA2}$, also from a *Bradyrhizobium*-like organism (Hill et al., 1999). Previous studies have shown little congruence between phylogenies based on the 16S rRNA gene and dehalogenase genes (Marchesi and Weightman, 2003b); however, given the association of *dehI* genes from this study with $dehI_{DA2}$, this clade apparently represents dehalogenases specifically associated with *Bradyrhizobium* sp. *Bradyrhizobium* isolates utilising several haloalkanes have also been reported previously (Sato et al., 2005; Sfetsas et al., 2009).

Given the presence of several distinct *dehI* clusters (three of which contained clones amplified from cDNA libraries, implying gene activity), all of which were associated with the same *Bradyrhizobium* phylotype, then these genes may have been acquired by horizontal gene transfer (HGT) during the lag phase. It has previously been shown that HGT can distribute catabolic genes, including dehalogenases, among different members of a bacterial community (Newby et al., 2000; Hill and Weightman, 2003), which may contribute to the length of the lag phase as a community adapts to degrade a novel compound. However, previous studies investigating the acclimation of environmental samples such as lake waters, sewage and sediments to degrade xenobiotic chemicals have argued that acclimation periods of reproducible duration among replicate experiments are not the result of HGT (Wiggins et al., 1987; Linkfield et al., 1989; Becker et al., 2006). As the acclimation period was very

reproducible between replicates in this study, it seems unlikely that a HGT event was responsible for the delayed onset of TCA dechlorination. As such, it is more probable that the *Bradyrhizobium* phylotype contained more than one dehalogenase within its genome, which is a common feature among previously characterised αHA degrading organisms (Slater et al., 1979; Klages et al., 1983; Janssen et al., 1985; Keuning et al., 1985; Barth et al., 1992).

Another organism similar to the facultative methylotroph *Methylibium petroleiphilum* PM1 (Nakatsu et al., 2006), was enriched during the latter stages of TCA dechlorination in the 30 mg/l tests (Figure 5.2D; Table 5.1). As well as utilising methanol as the sole carbon and energy source, *Methylibium petroleiphilum* PM1 is able to degrade numerous aromatic compounds such as benzene, toluene and phenol. This organism was putatively linked to the expression of a *dehII* gene (Figure 5.4) as neither the *Methylibium* sp. nor *dehII* expression was detected in any of the other tests. A similar phylotype (96% nucleotide sequence identity), also associated with the presence and expression of a *dehII* gene, was detected during 2MCPA degradation in Chapter 3.

The *dehI* genes present during TCA dechlorination in this study were all assigned to subgroup B (Figure 5.5). All the DehI enzymes assigned to subgroups A and B identified to date share the ability to dechlorinate both the D- and L-isomers of 2MCPA, whilst those from subgroups C and D act exclusively on the D-isomer. Most of the previously identified dehalogenases showing activity against TCA also showed activity against D- and L-2MCPA, and are also members of subgroups A and B; e.g. DehE from a *Rhizobium* sp. (Stringfellow et al., 1997), DehI of *P. putida* strain PP3 (Slater et al., 1979; Weightman et al., 1992) and DL-DEX from *Pseudomonas* sp. 113 (Motosugi et al., 1982). The association of the D- / L-enantiospecific dehalogenases (e.g. those in subgroups A and B) with TCA in this, and in previous studies, may indicate an inability of the D- specific DehI enzymes to dechlorinate TCA.

There was no detectable *dehII* expression in the 300, 120 or 60 mg/l tests, suggesting that the observed TCA dechlorination was not due to the presence of DehII enzymes within these RBTs. However, *dehII* expression (transcription) was detected in the 30

mg/l tests (Figure 5.4). Despite the diversity of *dehII* genes present in the RBTs (Figure 5.6), very little clustering of identical genes, which would be indicative of an enriched dehalogenase gene, was observed. The only *dehII* gene showing any sign of enrichment during TCA dechlorination was *dehII* cluster 1 (Figure 5.6). However, since *dehII* transcripts were too weak for cloning and sequencing, it is uncertain whether this dehalogenase was actively associated with TCA dechlorination.

Whilst there are no previous reports of DehII enzymes with activity against TCA, an earlier study identified DCA as the product of abiotic dechlorination of TCA in the presence of activated charcoal (Egli et al., 1989), which, given the previous association between DehII enzymes and DCA activity (Weightman et al., 1979; Chapter 3), might be considered to explain the observed *dehII* expression during TCA dechlorination in this study. However, since abiotic degradation of TCA was observed in an anaerobic system, and abiotic degradation was not detectable in this study, this explanation seems unlikely. Whilst the activity of a DehII with TCA can not be ruled out, it is possible that DehII expression was gratuitously induced by TCA in the 30 mg/l sludge tests, or that a *dehII* gene was co-transcribed as part of a polycistronic mRNA, which also contained a *dehI* gene encoding an enzyme with activity against TCA. The gene cluster containing the *dehI*° and *dehII* genes from *P. putida* PP3 is transcribed as a polycistronic (operonic) mRNA, as proposed by Dodds (2003) and later confirmed (M. J. Leggett and A. J. Weightman, unpublished results). It is therefore possible that the presence of *dehII* in the 30 mg/l RBTs was simply a consequence of induction of a *dehI* gene by TCA.

The rate of TCA dechlorination was markedly slower at the lowest sludge concentration, compared to tests conducted at higher concentrations, indicating that growth of the *Bradyrhizobium* phylotype may not have been directly associated with TCA degradation, but took place by cometabolism. Cometabolism in the strictest sense is the ability of an organism to transform non-growth-supporting substrates in the presence of a growth-supporting substrate (Arp et al., 2001). Weightman et al. (1992) demonstrated that additional readily degradable substrates were required to totally dechlorinate TCA. From initially rapidly dechlorinating TCA enrichments using soil inocula, they observed significantly lower rates of TCA dechlorination from

each successive subculture, which they concluded was due to carry over of metabolisable substrates from the soil, that were diluted out on subculturing. Other studies have also suggested that TCA degradation could only be accomplished in the presence of additional carbon sources or cofactors (Jensen, 1957a, 1960; Kearney et al., 1969), which may account for the lack of reported TCA degrading bacterial isolates in the literature. Indeed, all previous attempts at isolating TCA degrading organisms using TCA as the sole source of carbon and energy in our lab have proven unsuccessful. It is therefore possible that the reduced rate of TCA degradation at the lower inoculum concentration in this study reflects a reduction in concentration of essential co-factors, or additional readily degradable carbon sources present in the inoculum, thus restricting the growth of the TCA-degrading organism and consequently, reducing the rate of TCA dechlorination. Birch and Fletcher (1991) suggested that at the standard RBT inoculum concentration (30 mg/l), as much as 15 mg/l additional carbon is added to the test system. Therefore, tests conducted at 300 mg/l activated sludge could contain approximately 150 mg/l of additional readily degradable carbon, 7.5-times more carbon than is represented by TCA, the test substrate.

The OECD RBT protocols impose a maximum inoculum concentration of 30 mg/l (OECD, 1992). There are two reasons for this, both related to the presence of additional carbon in the test system. Firstly, RBTs utilise non-specific methods, such as dissolved organic carbon levels or the evolution of CO_2, to monitor substrate degradation. If significant quantities of additional carbon are added to the test along with the inoculum, it becomes difficult to distinguish between degradation resulting from the test substrate, and the endogenous carbon utilisation from the inoculum. Since a specific measure of degradation, dechlorination, was used in this study, this was not a problem. Secondly, test compounds for the RBTs should be present as the nominal sole source of organic carbon (OECD, 1992), which would certainly not be the case at higher sludge concentrations.

The degradation mechanism of TCA has not been well characterised. Oxalic acid has been identified as the product of TCA dechlorination (Motosugi et al., 1982; Ellis et al., 2001), which is presumably formed by a mechanism similar to the formation of glycollate and glyoxylate following dehalogenation of MCA and DCA, respectively.

However, other studies failed to detect oxalate and identified CO as a possible product following dechlorination of TCA, in cell free systems, by group I αHA dehalogenases (Weightman et al., 1992; Stringfellow et al., 1997). If CO were the product of TCA dechlorination, then any organism using TCA as a growth substrate would, by definition, have to be an autotroph. CO is metabolised aerobically by many bacteria, catalysed by aerobic carbon monoxide dehydrogenase (CODH) enzymes (King and Weber, 2007). There are two different CODH gene families, form I and form II. The form I family are considered to be true CODHs, whereas form II CODH are more tentatively associated with CO metabolism, and have been identified predominantly on the basis of sequence matches with bacterial genomes (King and Weber, 2007). Given the proposed association between TCA metabolism and CO utilisation outlined above, all TCA RBTs were assayed for the presence of form I and form II CODHs using PCR primers targeting the gene encoding the large subunit of the CODH enzyme, *coxL* (King, 2003). All tests contained PCR products of the correct sizes amplified using both form I and form II *coxL* gene primer sets (Figure 5.3); however, since *coxL* transcription was not assayed (using RT-PCR), their presence could not be definitively linked with TCA degradation. It should also be noted that the 16S rRNA gene sequence from the *Bradyrhizobium*-like phylotype enriched during TCA dechlorination in all test systems, was also identical to that of an *Afipia* sp. (formerly *Pseudomonas carboxydohydrogena* DSM; Anzai et al., 2000), a CO-utilising bacterium (Meyer et al., 1980) which contains a form I *coxL* gene (King and Weber, 2007). As the *Bradyrhizobium* sp. identified in the TCA RBTs from this study was tentatively associated with both dehalogenase and *coxL* genes, it is possible that it could grow on TCA, should CO be produced following its initial dehalogenation. Assuming bacteria capable of growth on TCA require both a DehI and a CODH, it might be considered that the lag phase could represent the time taken for HGT to bring these two genes into the same host; however, for the reasons outlined above, this is unlikely to have been the case in this study.

At lower sludge concentrations, the bacterial community took longer to acclimate to the presence of TCA, and also showed a reduced rate of TCA degradation. These results may be attributed to lower levels of degrading organisms, or reduced potential

for cometabolism, or HGT at lower sludge concentrations, or a combination of these factors.

This study suggest that current RBTs do not allow sufficient time for community acclimation, as TCA was rapidly degraded following a lag phase in all tests conducted at inoculum concentrations above 30 mg/l. Similarly, the limited inoculum concentration required by the RBT (maximum of 30 mg/l activated sludge) reduced the rate at which TCA was degraded. Current RBT protocols necessarily use low inoculum concentrations (30 mg/l activated sludge) due to the non-specific methods used to monitor substrate mineralisation; however, this limited inoculum size reduces the abundance of degrading organisms introduced into the RBT system, and also limits the possibility of cometabolic substrate degradation, which does not accurately reflect the situation in the environment. Clearly, TCA failed using the standard RBT, but with only twice the inoculum concentration and slightly enhanced acclimation period, TCA would have been passed as readily degradable in a modified RBT, as used in this study. In order to produce an improved reflection of the degradability of TCA without increasing the inoculum concentration, the test period would need to be extended slightly to better allow for the lengthy adaptation phase. Alternatively, pre-exposure of the inoculum to the test substrate prior to testing would enhance the numbers of specific degraders within the system, without significant additional carbon contamination, as has been suggested previously (van Ginkel et al., 1995; Thouand et al., 1996), thus reducing the lag phase prior to detectable substrate degradation.

Molecular genetic analysis indicated that a narrower range of dehalogenases (*dehI* only) and bacterial species were capable of TCA degradation, relative to those degrading 2MCPA in Chapter 4. It appears likely that dehalogenase activity against TCA may be restricted to DehI enzymes, and more specifically to those enzymes belonging to *dehI* subgroups A and B.

6. Isolation and Characterisation of Two New α-Halocarboxylic Acid Degrading Bacteria: *Pseudomonas* sp. Strain ML1 and *Rhodococcus* sp. Strain R1

6.1 Introduction

A key step in the detoxification of halogenated xenobiotics is the hydrolytic cleavage of the carbon-halogen bond by bacterial dehalogenases. A multitude of bacterial dehalogenases have been described previously with a variety of different substrate ranges and mechanisms of action (Fetzner and Lingens, 1994; Janssen et al., 1994). One group of intensively studied bacterial dehalogenases is the α-halocarboxylic acid (αHA) dehalogenases. Many bacteria able to grow on αHAs have been enriched, isolated and identified (Senior et al., 1976; Janssen et al., 1985; Tsang et al., 1988; Quamrul Hasan et al., 1994), and their cognate αHA dehalogenases characterised by various DNA and protein X-ray crystallographic analyses, shedding light on their structures and mechanisms of action (Hisano et al., 1996; Ridder et al., 1997; Ridder et al., 1999; Schmidberger et al., 2008).

From the pure culture work outlined above, and a limited amount of cultivation-independent work (Marchesi and Weightman, 2003a; Marchesi and Weightman, 2003b) it has become clear that currently known αHA dehalogenases are almost exclusively confined to the *Proteobacteria*. One notable exception is the recently identified haloacid dehalogenase from the thermophilic archaeon *Sulfolobus tokodaii* strain 7 (Rye et al., 2009). Though several reports have identified Gram Positive bacteria apparently capable of αHA dehalogenation (Kearney et al., 1963; Kerr and Marchesi, 2006), only one presented any data from biochemical analysis of the dehalogenase responsible (Chiba et al., 2009). The apparent lack of Gram Positive αHA dehalogenating organisms is especially surprising, given that other families of dehalogenase have been identified within *Actinobacteria*, such as haloalkane dehalogenases in *Arthrobacter* sp. (Scholtz et al., 1987), *Rhodococcus* sp. (Sallis et al., 1990) and *Corynebacterium* sp. (Yokota et al., 1987).

Two new αHA dehalogenating organisms, *Pseudomonas* sp. strain ML1 and *Rhodococcus* sp. strain R1 were isolated during investigations contributing to this

thesis. They were characterised in terms of their halogenated substrate utilisation, dehalogenase activities and dehalogenase gene sequences.

6.2 Results

6.2.1 Isolation of αHA-utilising bacteria

Two novel bacteria were isolated capable of utilising 2-monochloropropionic acid (2MCPA) as the sole source of carbon and energy. The first was isolated from an activated sludge inoculum (30 mg/l), using the modified RBT protocol described in Chapter 2, and designated strain ML1. The second was isolated from soil (0.8% w/v) using the same modified RBT protocol and designated strain R1.

Samples were taken from modified RBTs (following 100% dechlorination) and repeatedly subcultured in SBS medium containing 2MCPA (5 mM) as the sole source of carbon and energy. Following complete dechlorination of a subculture, samples were taken and cultured on SBS 2MCPA plates, and selected colonies were purified via cultivation back into liquid media. This process was repeated until cultures were pure (checked by cultivation on nutrient agar). One isolate was selected for further study from the activated sludge inoculum, whilst 6 similar isolates (designated strains R1-R6) were chosen from the soil inoculum. Random amplified polymorphic DNA (RAPD; See Chapter 2) analysis of all 6 isolates showed them to be identical (results not shown) and therefore, only strain R1 was chosen for further analysis.

6.2.2 Growth characteristics of the bacterial isolates

Strain ML1 grew on 2MCPA at 30°C with a specific growth rate of 0.15 h^{-1} whilst strain R1 grew with a specific growth rate of 0.38 h^{-1}, both with concomitant release of Cl^- during growth (Figure 6.1). Strain ML1 also utilised acetate and dichloroacetic acid (DCA) as growth substrates, and weakly (20%) dechlorinated monochloroacetic acid (MCA), but showed no significant growth (according to the optical density of the cultures) with any of the other halogenated substrates tested (Table 6.1). When provided with an additional carbon source (acetate), strain ML1 was also able to totally dechlorinate MCA, partially dechlorinate (69%) monobromoacetic acid (MBA), and maintained its activity towards DCA and 2MCPA; however, growth was apparently inhibited by 2-monobromobutyric acid (2MBBA).

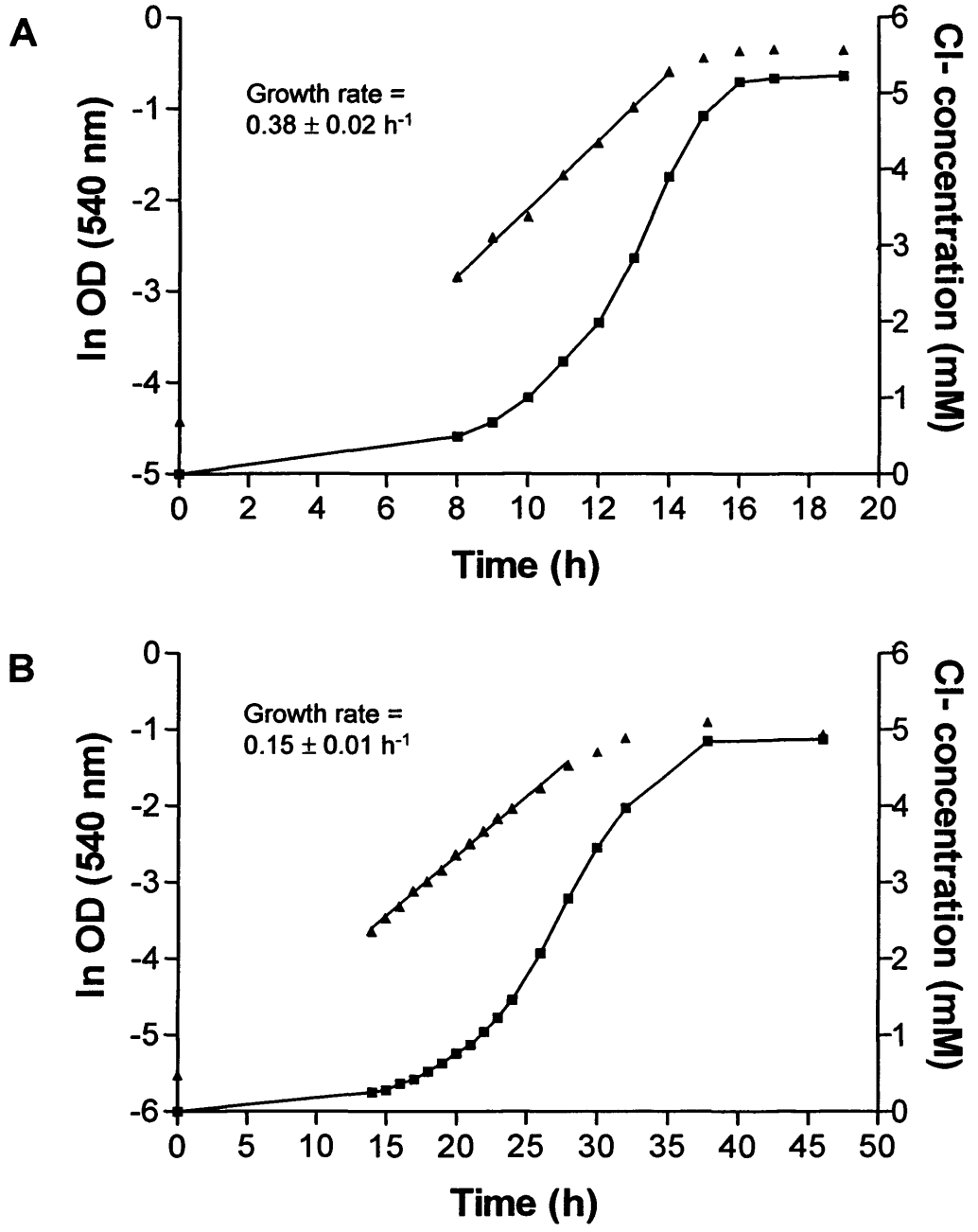

Figure 6.1 Indicative growth and dechlorination curves for *Rhodococcus* sp. strain R1 and *Pseudomonas* sp. strain ML1. Optical density = (▲), Cl^- release = (■). (A) *Rhodococcus* sp. strain R1; and (B) *Pseudomonas* sp. strain ML1, grown on 5 mM SBS 2MCPA at 30°C. Growth rate (h^{-1}) values indicated are mean values (n = 3) ± the standard deviation. Error bars, representing the standard error of the mean (n = 2) for Cl^- values are too small to see within the symbols shown.

Table 6.1 Substrate utilisation profile for *Pseudomonas* sp. strain ML1 and *Rhodococcus* sp. strain R1 grown in the presence of various halogenated substrates with or without acetate as an additional carbon source

Substrate	% Substrate dehalogenation[a,b]			
	Pseudomonas sp. ML1		*Rhodococcus* sp. R1	
	Halogenated substrate only	Halogenated substrate plus acetate	Halogenated substrate only	Halogenated substrate plus acetate
MCA	20 (0.15)	100 (1.75)	8 (0.00)	29 (0.08)
DCA	100 (0.61)	100 (2.62)	4 (0.03)	3 (2.64)
TCA	2 (0.08)	2 (0.83)	3 (0.05)	3 (0.85)
MBA	7 (0.07)	69 (1.00)	10 (0.00)	93 (0.08)
2MCPA	100 (1.60)	100 (3.18)	100 (1.79)	100 (3.67)
22DCPA	5 (0.12)	5 (0.85)	19 (0.01)	86 (1.02)
2MCBA	3 (0.04)	4 (0.67)	3 (0.04)	12 (0.10)
2MBBA	2 (0.00)	0 (0.07)	2 (0.03)	0 (0.12)

a. Values represent substrate dehalogenation over a 1-4 day period. b. Values in the parenthesis show max OD_{540} observed from halogenated substrate cultures relative to max OD_{540} observed from acetate cultures.

Strain R1 only utilised 2MCPA as the sole source of carbon and energy, and whilst it also weakly dechlorinated (19%) 2,2-dichloropropionic acid (22DCPA), this was not coupled to any significant growth. In the presence of acetate as an additional carbon source, strain R1 maintained its ability to grow on 2MCPA, and also strongly dechlorinated MBA (93%) and 22DCPA (86%), as well as dechlorinating MCA (29%), but no significant growth was observed with either MCA or MBA.

6.2.3 Dehalogenase activity of cell-free extracts (CFE) from strains ML1 and R1

Cell free extracts (CFE), prepared from either strain ML1 or strain R1 cultures grown on SBS acetate and 2MCPA, were assayed for activity with MCA, DCA and 2MCPA (Table 6.2). CFE from strain ML1 showed high activity with DCA (0.25 U/mg protein), followed by MCA (0.15 U/mg protein) and then 2MCPA (0.03 U/mg protein). CFE from strain R1 were most active with MCA (0.08 U/mg protein) > 2MCPA (0.07 U/mg protein) > DCA (0.01 U/mg protein). No significant activity was detected within the cell debris removed from the crude cell extract by centrifugation following breakage of cells in the French pressure cell (see section 2.3; results not shown).

Native PAGE of CFEs from strains ML1 and R1, stained with αHA substrates, allowed the visualisation of dehalogenase activity; a CFE prepared from *P. putida* strain PP3 was included for comparison. Two active dehalogenases were present in the CFE from strain ML1 (Figure 6.2A), which co-migrated with those from *P. putida* strain PP3 suggesting a similarity between these enzymes. One of the two dehalogenases, ($DehI_{ML1}$; Figure 6.2A and B) showed stereospecific dechlorination of D- but not L- 2MCPA (lane 2, Figure 6.2B and C). The second enzyme from strain ML1, $DehII_{ML1}$ (Figure 6.2) appeared to show higher activity with L-2MCPA. CFEs from strain R1 showed only a single dehalogenase with different electrophoretic mobility than those from strains PP3 and ML1. This enzyme showed activity with MCA and both D- and L-2MCPA (Figure 6.2).

Table 6.2 Specific dehalogenase activities of cell-free extracts with various αHA substrates.

Substrate	Specific activity (U/mg protein)[a,b]	
	***Pseudomonas* sp. ML1**	***Rhodococcus* sp. R1**
MCA	0.15 (1.0)	0.08 (1.0)
DCA	0.25 (1.7)	0.01 (0.1)
2MCPA	0.03 (0.2)	0.07 (0.9)

a. A unit of dehalogenase activity is defined as the amount of enzyme required to convert 1 μmol substrate / min. b. Values in parenthesis represent specific activity relative to MCA

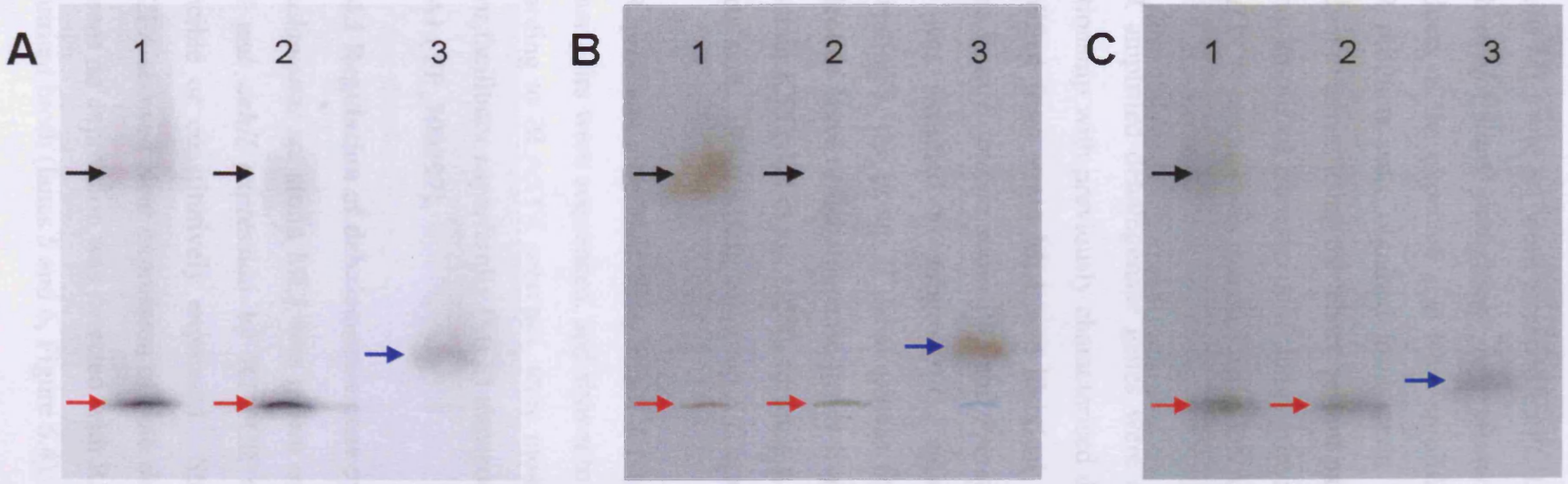

Figure 6.2 Native PAGE of cell free extracts incubated with various substrates: (A) MCA; (B) D-2MCPA; or (C) L-2MCPA, to show dehalogenase activity. Lane numbers: (1) *P. putida* PP3. (2) *Pseudomonas* sp. ML1. (3) *Rhodococcus* sp. R1. Black arrows mark the position of $DehI_{PP3}$ for *P. putida* PP3 and a putative D-specific DehI ($DehI_{ML1}$) from strain ML1. Red arrows mark the position of $DehII_{PP3}$ and a putative DehII ($DehII_{ML1}$) from strain ML1. Blue arrows represent a putative D-/L- dechlorinating dehalogenase (Deh_{R1}) from strain R1.

6.2.4 Identification and characterisation of *dehI* and *dehII* genes present in strains ML1 and R1

Strains ML1 and R1 were screened by PCR for the presence of *dehI* and *dehII* genes. Both *dehI* ('short' and 'long' *dehI* primer sets; see section 2.6.3) and *dehII* PCR products of the expected size were amplified from strain ML1 (results not shown). PCR products were obtained from strain R1 using both *dehI* primer sets, but the product obtained using the 'short' primer pair was larger than expected, similar in size to that amplified from the *dehI* 'long' primer set (c.a. 500 bp; results not shown). No *dehII* PCR product was detected using DNA from strain R1.

PCR amplified dehalogenase genes were cloned and sequenced to determine their relationship with previously characterised dehalogenases. The group I dehalogenase amplified from strain ML1 was identical at the nucleotide sequence level to *dehI* genes from *P. putida* strain PP3 and *Pseudomonas* sp. strain K55 (Hill et al., 1999) and was assigned to subgroup C, which also contains *hadD* (Figure 6.3A). Interestingly, the group II dehalogenase from strain ML1 was also identical at the nucleotide level to dehalogenase genes from *P. putida* strain PP3 and *Pseudomonas* sp. strain K55 (Hill et al., 1999), forming a strongly supported clade along with *hadL* (Barth et al., 1992) within subgroup A (Figure 6.3B).

PCR products amplified from strain R1 using both the long and short *dehI* PCR primer pairs were sequenced, and shown to amplify a highly similar fragment, which according to BLASTX searches, were most similar (~70% amino acid identity) to a major facilitator superfamily (MFS) transporter protein from *Rhodococcus jostii* strain RHA1 (YP_700397).

6.2.4.1 Regulation of dehalogenase gene expression in strain ML1

Pseudomonas sp. strain ML1 was grown on various different media and assayed for *dehI* and *dehII* expression by RT-PCR to determine whether these genes were inducible or constitutively expressed. Strain ML1 grown with SBS acetate and 2MCPA showed clear expression of both *dehI* and *dehII* (lane 4, Figure 6.4A and B), whereas no expression was detected with RNA from cultures grown on either acetate or nutrient broth (lanes 5 and 6, Figure 6.4).

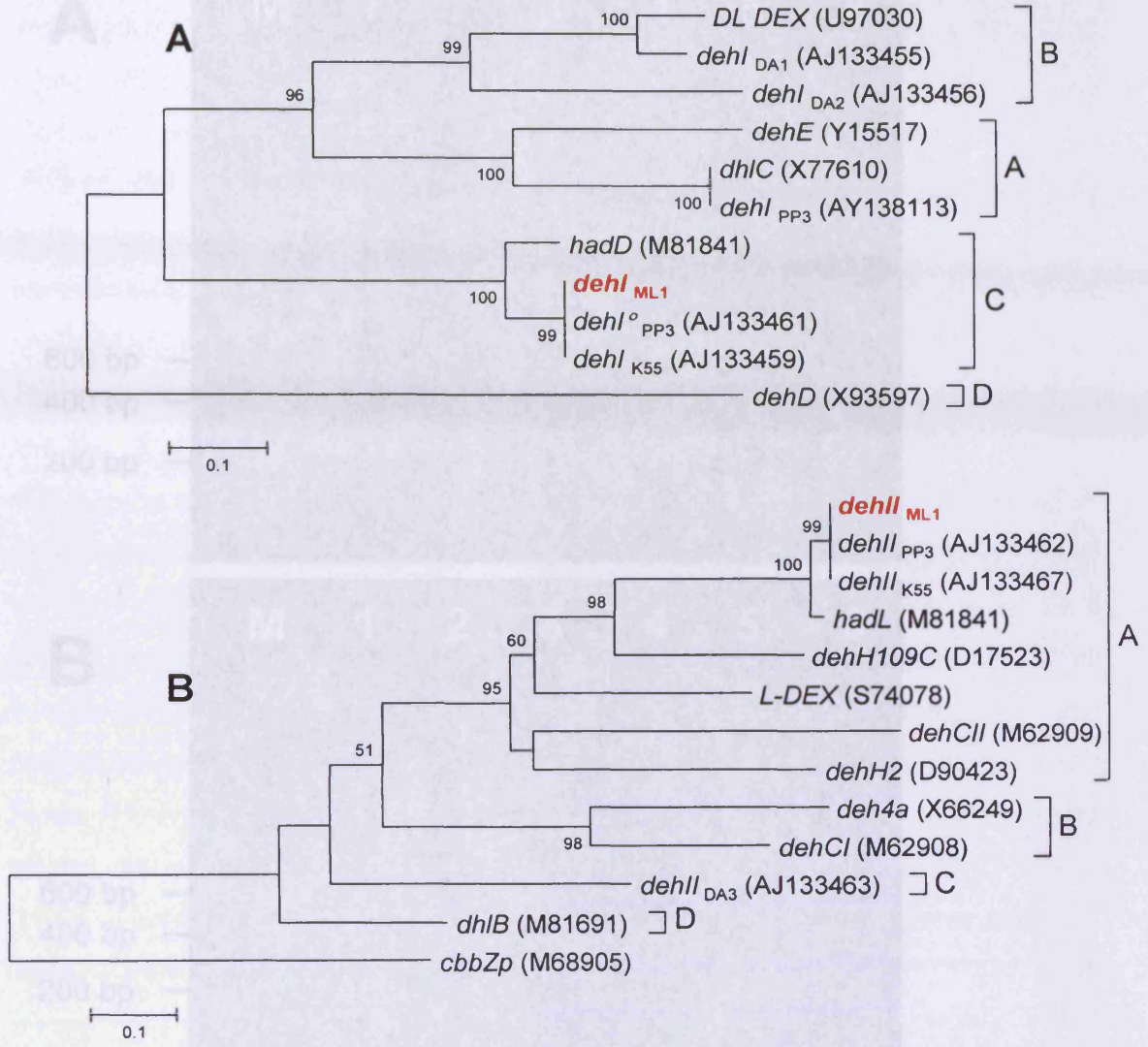

Figure 6.3 Dendrogram illustrating the relationship between the dehalogenase genes of *Pseudomonas* sp. strain ML1 and references from the database: (A) *dehI*$_{ML1}$ and other *dehI* genes from various organisms based on an alignment of 427 nucleotides. (B) *dehII*$_{ML1}$ and other *dehII* genes from various organisms, based on an alignment of 368 nucleotides. Trees were 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. Scale bar represents the number of base substitutions per site.

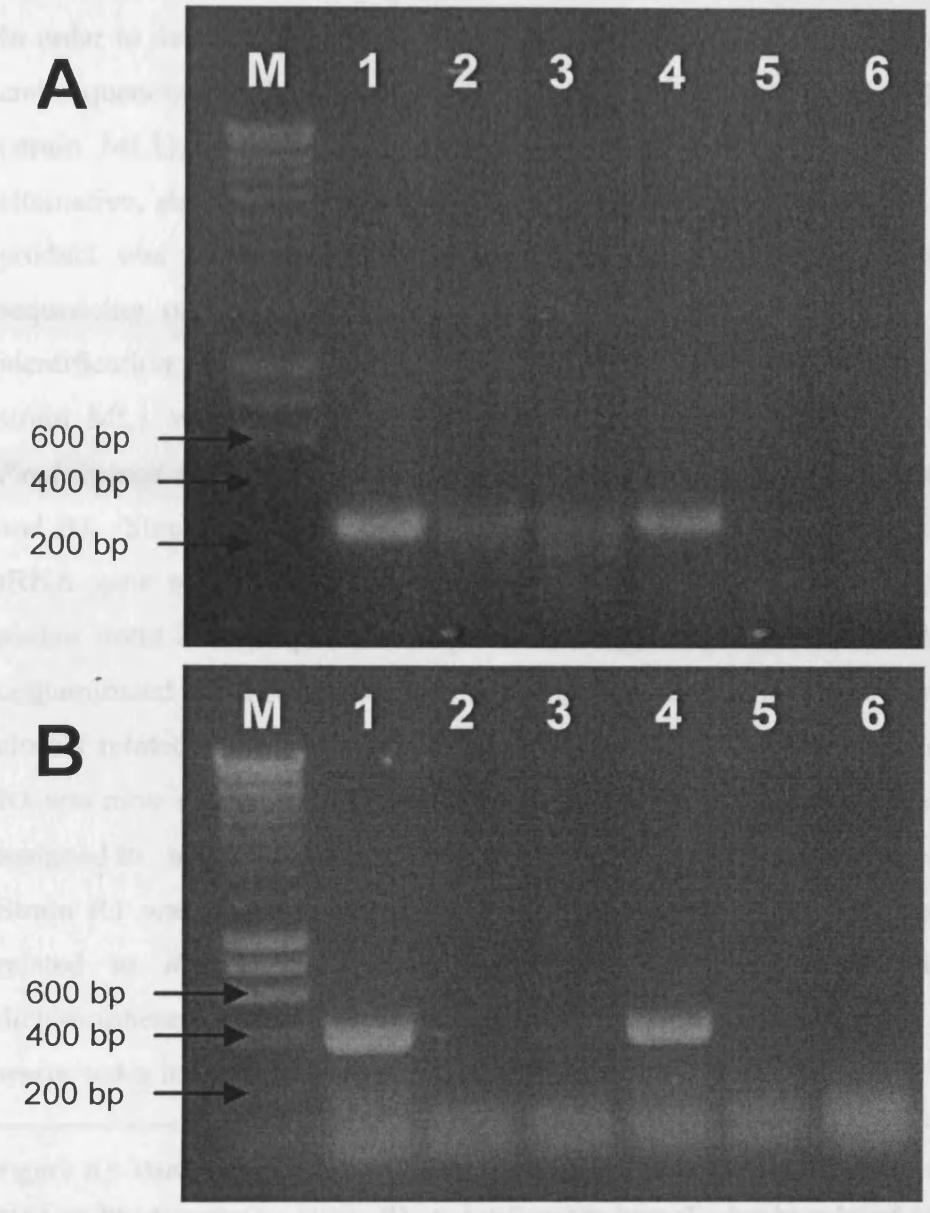

Figure 6.4 Agarose gel showing expression (transcription) of *dehI* and *dehII* genes amplified from *Pseudomonas* sp. strain ML1 and *P. putida* strain PP3. (A) *dehI* RT-PCR products; and (B) *dehII* RT-PCR products; amplified from *P. putida* strain PP3 as a control (lanes 1-3) and *Pseudomonas* sp. strain ML1 (lanes 4-6) grown on various growth media: SBS acetate + 2MCPA (lanes 1 and 4); SBS acetate (lanes 2 and 5); nutrient broth (lanes 3 and 6). M = marker.

6.2.5 Phylogenetic affiliation of strains ML1 and R1

In order to determine the closest phylogenetic relatives of each isolate, amplification and sequencing of the 16S rRNA gene, positions 27 – 907 (strain R1) or 27 – 1492 (strain ML1), were used to determine their closest phylogenetic relatives. An alternative, shorter region of the 16S rRNA gene was amplified for strain R1 as no product was obtained for the primer pair 27F and 1492R. Amplification and sequencing of the DNA gyrase subunit B gene (*gyrB*) was also used to assist identification of strain ML1. In both 16S rRNA gene and *gyrB* phylogenetic trees, strain ML1 was assigned to highly supported clades containing only fluorescent *Psedomonas* spp., and was distinct from other *Gammaproteobacteria* (Figure 6.5A and B). Strain ML1 was therefore named *Pseudomonas* sp. strain ML1. The 16S rRNA gene sequence of *Pseudomonas* sp. strain ML1 was identical to that of *P. putida* strain PC36, a phenol and *p*-creosol degrading organism isolated from phenol contaminated water (Merimaa et al., 2006), whereas the *gyrB* gene sequence was most closely related to *P. fluorescens* strain ATCC 17467 (95% sequence identity). Strain R1 was most similar to 16 rRNA gene sequences from the *Actinobacteria*, and was assigned to a clade composed exclusively of *Rhodococcus* sequences (Figure 6.5C). Strain R1 was therefore named *Rhodococcus* sp. strain R1, and was most closely related to *R. koreensis* strain DNP505 (98.5% nucleotide identity), a 2,4-dichlorophenoxyacetic (2,4-D) acid degrading *Rhodococcus* isolated from industrial waste water in Korea (Yoon et al., 2000).

Figure 6.5 Dendrogram illustrating the phylogenetic relationships of *Pseudomonas* sp. strain ML1 or *Rhodococcus* sp. strain R1, and references from the database based on an alignment of the 16S rRNA or *gyrB* genes. A) Dendrogram illustrating the phylogenetic relationship between *Pseudomonas* sp. strain ML1 and 18 other strains from the database (*P. putida* strain PP3 was sequenced in this study) based on an alignment of 1267 nucleotides from the 16S rRNA gene. B) Dendrogram illustrating the phylogenetic relationship between *Pseudomonas* sp. strain ML1 and 16 other strains from the database (*P. putida* strain PP3 was sequenced in this study) based on an alignment of 1075 nucleotides from the *gyrB* gene. C) Dendrogram illustrating the phylogenetic relationship between *Rhodococcus* sp. strain R1 and 13 other strains from the database based on an alignment of 788 nucleotides from the 16S rRNA gene. All trees were 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. Isolates sequenced in this study are shown in bold. T = type strain. Scale bar represents the number of base substitutions per site.

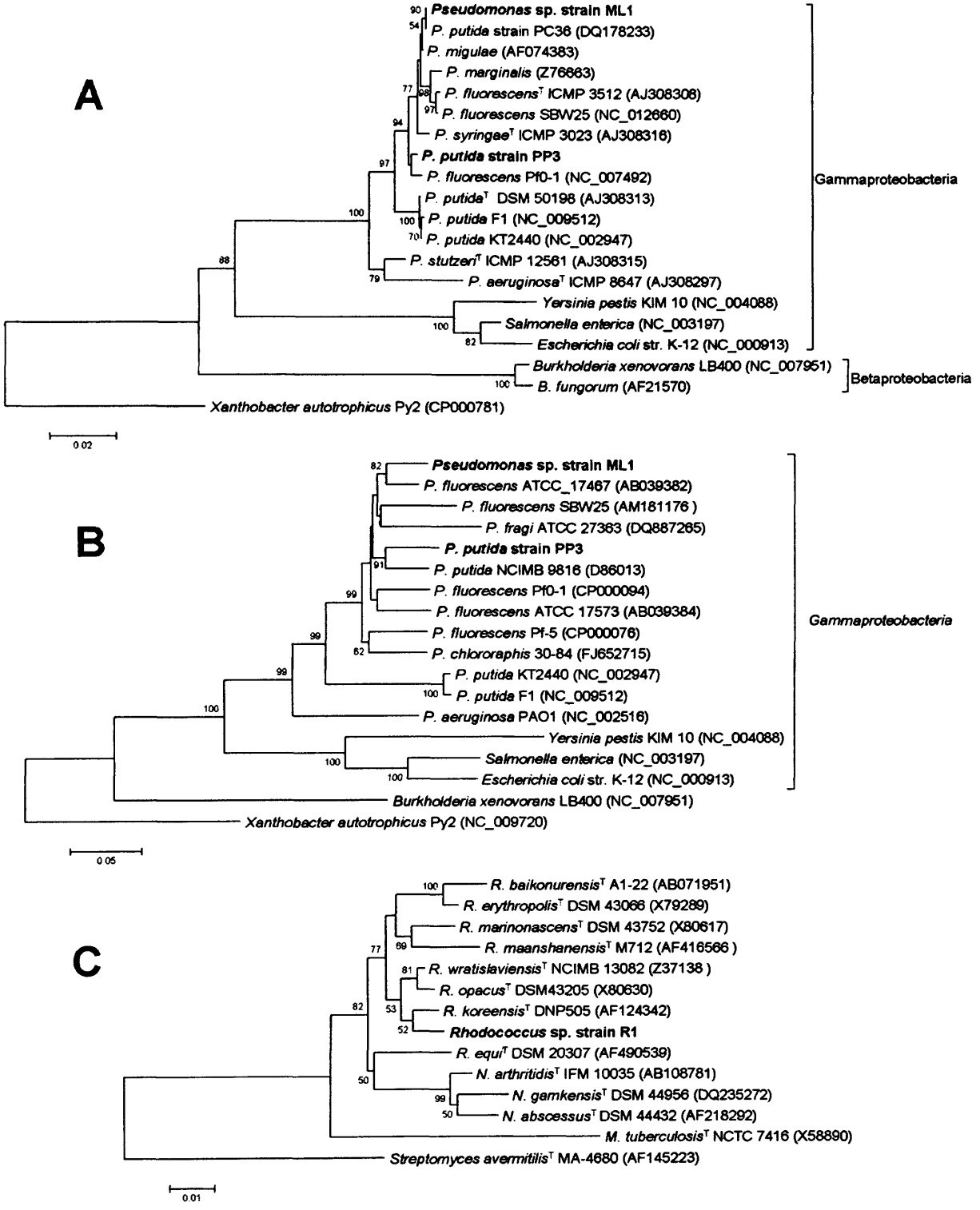

Figure 6.5 For legend, see previous page.

6.3 Discussion

Two new bacteria capable of growing on the αHA 2MCPA were isolated and partially characterised. Strain ML1 was identified as a *Pseudomonas* sp. based on the nucleotide sequences of both the 16S rRNA gene and the *gyrB* gene (Figure 6.5A and B). The two trees are not highly congruent; ML1 being grouped most closely with a *P. putida* and *P. fluorescens* for the 16S rRNA and *gyrB* analysis, respectively. However, previous studies have shown that both the *P. putida* and *P. fluorescens* lineages, in particular, are difficult to resolve, especially on the basis of 16S rRNA gene sequence alone, and even in combination with several other genes (Yamamoto and Harayama, 1998; Yamamoto et al., 2000; Hilario et al., 2004). For definitive identification to the species level, it will therefore be necessary to use other methods to complement the 16S rRNA gene based classification reported here, employing a polyphasic approach including determination of chemotaxonomic characteristics such as fatty acid, protein or polar lipid profiles (Moore et al., 2006; Peix et al., 2009). Isolate R1 was identified as a member of the family *Nocardiaceae* of the *Actinobacteria*, and tentatively assigned to the genus *Rhodococcus* based on its 16S rRNA gene sequence (Figure 6.5C). The phylogeny of actinomycete species, and especially the closely related *Rhodoccoccus* and *Nocardia* genera, is also difficult to delineate accurately on the basis of their 16S rRNA gene sequence alone (Gürtler et al., 2004), and therefore this identification will require further confirmation, possibly using phenotypic properties (Goodfellow et al., 1998; Goodfellow and Maldonado, 2006), or using the genomic methodology suggested by Gürtler et al. (2004). Regardless of its specific phylogenetic identification, strain R1 represents the first actinomycete, and one of the only Gram Positive organisms identified able to utilise αHAs.

Both *Pseudomonas* and *Rhodococcus* species share a capacity to degrade a wide variety of organic compounds, including many halogenated xenobiotics. *Pseudomonas* spp. are catabolically versatile organisms capable of degrading a wide range of aliphatic and aromatic hydrocarbons, as well as numerous halogenated derivatives, including chlorinated biphenyls (Parsons et al., 1988), bromo- chloro- and fluorobenzoates (Engesser and Schulte, 1989; Komancová et al., 2003), and also, many αHA (Slater et al., 1979; Motosugi et al., 1982; Klages et al., 1983; Hasan et

al., 1994). Rhodococci are capable of degrading some of the most recalcitrant substrates, including long and short chain alkanes, aromatic, heterocyclic and polycyclic aromatic compounds (Larkin et al., 2005). Many halogenated compounds are degraded by *Rhodococcus* spp., including mono-, di- and trifluorophenols (Bondar et al., 1998; Finkelstein et al., 2000), various chlorophenols, chlorobenzenes and polychlorinated biphenyls (Bondar et al., 1999; Vogt et al., 2004; Leigh et al., 2006) and some brominated phenols and benzenes (Zaitsev et al., 1995). *R. rhodochrous* NCIMB 13064 is capable of degrading several shorter chain (C_3-C_{10}) chloroalkanes, catalysed by the haloalkane dehalogenase DhaA (Kulakova et al., 1997), and whilst there is some preliminary evidence of *Rhodococcus* activity against β-halocarboxylic acids (Jing and Huyop, 2007), this study is the first to report a *Rhodococcus* sp. with activity against αHAs.

Both organisms had a relatively narrow αHA substrate range. ML1 was able to utilise 2MCPA and DCA as sole sources of carbon and energy, as well as MCA and MBA in the presence of acetate as an additional carbon source. Growth on DCA as a sole source of carbon and energy is uncommon in the literature, despite being readily degraded by environmental inocula (see Chapter 3 and Ellis et al., 2001); the only other recorded incidences being *Xanthobacter autotrophicus* GJ10 (Janssen et al., 1985), and several proteobacterial strains isolated from drinking water (Zhang et al., 2009a). Strain R1 only grew on 2MCPA as sole carbon source, the substrate on which it was isolated, though it also dechlorinated 22DCPA and MBA in the presence of acetate, as well as showing weak dehalogenating activity with MCA. Growth of both organisms on acetate was apparently inhibited by 2MBBA. 2MBBA, MCA, DCA and MBA have been shown previously to inhibit the growth of the dehalogenating organism *P. putida* strain PP3 (Weightman et al., 1985). Allison et al. (1983) also showed that monohalogenated acetates were potent growth inhibitors of a bacterium isolated on 22DCPA, which seems also to be the case for *Rhodococcus* sp. R1, where growth was apparently inhibited by MCA and MBA, as well as by 2MCBA.

PAGE zymography suggested that ML1 contained at lest two active dehalogenases, $DehI_{ML1}$ and $DehII_{ML1}$, which appeared very similar to dehalogenases produced by *P.*

putida PP3 (Figure 6.2). However, unlike $DehI_{PP3}$, $DehI_{ML1}$ was active against D-, but not L-2MCPA. There are only three confirmed cases of D-specific dehalogenase enzymes reported in the literature: DehD from a *Rhizobium* sp. (Cairns et al., 1996); HadD from *P. putida* strain AJ1 (Smith et al., 1990); and D-2-MCPA from *Rhizobium* sp. strain NHG3 (Higgins et al., 2005). Cloning and sequencing of PCR amplified *dehI* and *dehII* genes from strain ML1 showed them to be identical at the nucleotide sequence level to *dehI°* and *dehII* of *P. putida* strain PP3 (Figure 6.3; Hill et al., 1999). Interestingly, *dehI°*$_{PP3}$ is a silent gene within the genome of strain PP3, which has been expressed in *E. coli* and has been shown to have activity with D-, but not L-2MCPA (Hope and Weighman, unpublished), supporting the identification of $DehI_{ML1}$ as a D-specific *dehI*. $DehI_{PP3}$, the only functional DehI in strain PP3, was not detected in *dehI* clone libraries prepared from strain ML1, which may explain its inability to grow on 22DCPA, as the majority of 22DCPA activity in strain PP3 was due to $DehI_{PP3}$ (Weightman et al., 1979). However, strain PP3 is unable to grow on glyoxylate (Weightman et al., 1985), the product of DCA dechlorination, whereas ML1 was able to utilise DCA as a growth substrate, suggesting it has a glyoxylate degradation pathway. The presence of identical *dehI* and *dehII* genes in three separately isolated *Pseudomonas* sp. (strains PP3, K55 and ML1; Figure 6.3) suggests that horizontal gene transfer may be responsible for the distribution of these genes, which is consistent with the common association of dehalogenases with mobile genetic elements (Kawasaki et al., 1992; Thomas et al., 1992a; Weightman et al., 2002; Hill and Weightman, 2003).

Both *dehI* and *dehII* genes from ML1 were induced by the presence of 2MCPA, with no detectable expression of either dehalogenase when grown in the absence of 2MCPA (Figure 6.4). Previous studies have shown αHA dehalogenases to be under the control of specific regulators, such as the RpoN (σ^{54}) dependent regulator of *P. putida* PP3 (Thomas et al., 1992b; Topping et al., 1995), and associated with permeases, such as Deh4p, an MCA permease from *B. cepatia* MBA4 (Yu et al., 2007) and DehP from strain PP3 (Dodds, 2003).

Rhodococcus sp. strain R1 contained at least one active dehalogenase with distinct mobility from those of strain ML1 (Figure 6.2). PCR products were amplified from

strain R1 using both *dehI* primer sets, but both products were apparently of identical size (results not shown). Neither product showed any homology to previously characterised dehalogenase sequence, although they shared 70% amino acid identity with a major facilitator superfamily (MFS) transporter protein from *R. jostii* strain RHA1. The MFS is one of the largest families of membrane transporters on earth and are known to transport a wide variety of molecules, including "simple sugars, oligosaccharides, inositols, drugs, amino acids, nucleosides, organophosphate esters, Krebs cycle metabolites, and a large variety of organic and inorganic anions and cations" (Pao et al., 1998). An MFS family permease, Deh4p, is essential for the uptake of MCA in *Burkholderia cepatia* MBA4 (Yu et al., 2007), but there was no significant nucleotide or amino acid homology between Deh4p and the PCR product amplified from R1 (results not shown). Sequence analysis clearly showed two binding sites for the *dehI* forward primer, $dehI_{F1}$, at either end of the amplified fragment suggesting that only the forward primer was required for amplification, which explains why both *dehI* primer sets gave identical products.

PAGE zymography (Figure 6.2) suggests that the dehalogenase present in strain R1, designated Deh_{R1}, was able to dechlorinate both D- and L-2MCPA, an attribute found only in the group I αHA dehalogenases (Hill et al., 1999). However, since no *dehI* or *dehII* gene products were detected by PCR, this enzyme may represent a novel αHA dehalogenase gene. Future work will, therefore, aim to determine the nucleotide sequence of Deh_{R1} in order to assess its phylogenetic relationship to previously described dehalogenases. A dehalogenase with similar characteristics to Deh_{R1} (showing activity with both D- and L-2MCPA and not amplifiable using the group I or group II αHA dehalogenase primers; Hill et al., 1999) was recently identified in a *Bacillus* sp. from marine sediment (Chiba et al., 2009). If this enzyme is phylogenetically related to Deh_{R1}, then these two enzymes will represent a dehalogenase lineage currently only found in Gram Positive organisms.

The specific activities of CFEs from strain R1 were relatively low compared to strain ML1 (Table 6.2), which was surprising, given that strain R1 was able to totally dechlorinate 2MCPA with a much higher growth rate than strain ML1. This suggests that the enzyme assay may have been performed under sub-optimal conditions, and

that this dehalogenase may have a different pH optimum, as well as other characteristics that differ from previously described αHA dehalogenases.

7. General Discussion

The ability of bacteria to grow by utilising xenobiotic compounds as carbon and energy sources has been intensively studied for over 50 years (Jensen, 1957a; Dagley et al., 1964; Alexander, 1965; Reineke and Knackmuss, 1979; Pieper et al., 1988; Engesser and Schulte, 1989; Janssen et al., 1994; Spiess et al., 1995; Janssen et al., 2001). Whilst such studies have helped identify enzymes responsible for degradation and established metabolic pathways for many of these compounds there is increasing interest in the adaptation of bacterial communities during exposure to xenobiotics. More recently, some studies have investigated the relationship between microbial community composition, based on cultivation-independent analysis of PCR amplified 16S rRNA genes (Fernández et al., 1999; Ayala-del-Río et al., 2004; Becker et al., 2006), and community function (biodegradation) as reflected by catabolic gene analysis (de Lipthay et al., 2002; Marchesi and Weightman, 2003b; Flocco et al., 2009). However, few studies have performed a detailed analysis of both the whole community and catabolic gene pool during adaptation to degrade xenobiotic compounds. This study used a combination of cultivation-independent methods to characterise and monitor more comprehensively the bacterial community, and functional gene pool, during acclimation to degrade α-halocarboxylic acids (αHAs) with the aim of applying the understanding of community adaptation to improving upon standard OECD ready biodegradation test (RBT) methods.

The time taken for a community to become acclimated to a given compound varies tremendously according to the changes required within that population in order to accomplish substrate transformation or degradation. Therefore, the biodegradative potential and rate at which a community can adapt to degrade a test chemical is likely to have a significant effect on the classification of a chemical's biodegradability in the RBT, whilst test failure is generally linked to the inability of an inoculum to adapt to the test chemical of interest (Toräng and Nyholm, 2005). In this study, RBTs conducted using higher inoculum concentrations were more likely to achieve an RBT pass than those conducted at lower inoculum concentrations, suggesting that in general, larger inoculum size confers a greater ability for an inoculum to successfully adapt to, and degrade the test chemical. Although rarely demonstrated unequivocally, community acclimation to degrade a persistent chemical is generally considered to

involve one, or a combination of the following mechanisms (Linkfield et al., 1989; de Lipthay et al., 2002; Becker et al., 2006): i) induction of catabolic genes/enzymes; ii) mutation, genetic exchange (horizontal gene transfer - HGT) or rearrangement; iii) alleviation of unfavourable conditions within the inoculum, e.g. additional readily degradable carbon sources or substrate toxicity; or iv) enrichment of an organism or organisms from the inoculum to a level where biodegradation is detectable. The results from this study are considered in light of these mechanisms below.

7.1 Catabolic Gene Induction and Bacterial Acclimation To Xenobiotics

Whilst αHA dehalogenases are almost always detected within organisms or populations degrading αHAs (Hill et al., 1999; Hill and Weightman, 2003; Marchesi and Weightman, 2003a; Marchesi and Weightman, 2003b), this study is the first to detect dehalogenase gene expression (transcription) coincident with substrate dechlorination by a microbial community derived from activated sludge. The identification of catabolic gene transcription from RNA analysis is important because DNA analysis alone would detect both active and inactive microbial populations, as well as exogenous DNA free of microbial cells (Bodrossy et al., 2006; Nielsen et al., 2007). The time required for induction of catabolic genes and their translation into proteins is commonly cited as an explanation for the lag phase during microbial acclimation (Wiggins et al., 1987; Becker et al., 2006). Dehalogenase gene expression in this study only took place during substrate dechlorination, regardless of the substrate or inoculum concentration used, suggesting the induction of dehalogenase gene expression in response to an αHA stimulus. However, the effect of αHA dehalogenase gene induction on the outcome of model RBTs is likely to be minimal in comparison to other effects (discussed below), since the lag phases observed prior to substrate dechlorination were long (measurable in days), where enzyme induction would normally take only minutes or hours (Richmond, 1968). Therefore, induction of catabolic genes/enzymes is unlikely to be a significant factor effecting RBT outcome, and is unlikely to be influenced by inoculum size. However, as described below (section 7.3), gene expression (or repression) may in some cases be linked to delayed adaptation of an inoculum.

7.2 Genetic Mutation, Rearrangement and Horizontal Gene Transfer During Community Acclimation

Community adaptation by genetic mutation, rearrangement and/or HGT could conceivably impact on RBT outcome, most likely serving to delay the onset of substrate degradation. Genetic exchange involving the HGT of mobile genetic elements (MGE) is known to play a key role in the acclimation of a microbial population to degrade xenobiotics. For example, Dejonghe et al. (2000) clearly demonstrated that plasmid transfer was involved in the adaptation of a soil community to degrade the herbicide 2,4-D, whilst HGT has also been associated with bacterial adaptation to αHA degradation (Hill and Weightman, 2003). There was some evidence in the present study to suggest that HGT was in operation during adaptation to αHAs. The presence of different dehalogenase genes (clusters 3 and 4, Figure 4.4; clusters 7 and 8, Figure 4.5), tentatively associated with the same host organism (the *Starkeya* phylotype from tests 0.3A and 0.3B, Chapter 4 – Figure 4.2G and H, respectively; Table 4.1) suggest that this organism acquired different dehalogenase genes during acclimation. Similarly, the presence of different dehalogenases (Figure 5.5) all enriched in association with the same *Bradyrhizobium* sp. in Chapter 5 (Figure 5.2; Table 5.1), was suggestive of gene acquisition via HGT. Conversely, the high level of reproducibility of the lag phases during community adaptation in the TCA RBTs suggest this may not have been the case (Linkfield et al., 1989; Becker et al., 2006).

Bacteria may also "acquire" novel traits by activating silent genes already present within their genomes. This may have been the case in test 0.3B (Chapter 4) where a *dehI* gene enriched at the same time as *dehII* remained inactive until the community underwent a period of starvation (indicated by a temporary cessation of dechlorinating activity) at which point, *dehI* became activated (transcribed). Activation of silent genes in response to starvation stress has been reported previously and is commonly the result of increased insertion sequence (IS) activity (Matin et al., 1989; Hall, 1999b). Insertion sequence activity can cause gene activation in several ways. For instance the silent, promoterless phenol degradation genes, *pheBA*, can be activated by the adjacent insertion of IS*1411*, which contains an outwardly-directed promoter (Kallastu et al., 1998). IS are also involved in the activation of the *bgl* operon, where

silencer elements responsible for maintaining the *bgl* operon in its silent state, are insertionally inactivated, commonly by IS*1* or IS*5*, therefore activating the operon (Reynolds et al., 1981; Hall, 1999a).

However, we still know relatively little about when, where, between which species, and at what frequencies such mechanisms actually occur in the environment (Top and Springael, 2003), making it difficult to logically design the RBT conditions to allow for these processes to take place.

7.3 Optimisation of Conditions For Biodegradation

Another possible mechanism of community adaptation in the model RBTs in this study was the alleviation of conditions not conducive to biodegradation. The lag phase observed prior to TCA degradation (Chapter 5) may have resulted from the preferential utilisation of more readily degradable carbon sources in the test inoculum ahead of the test substrate, as was shown previously to delay the degradation of *p*-nitrophenol (Swindoll et al., 1988), 3-chlorobenzoate (Becker et al., 2006), and 4-chlorophenol (Kuiper and Hanstveit, 1984), possibly as a result of catabolite repression. Catabolite repression is a regulatory phenomenon where by the expression of genes for assimilation of secondary carbon sources are reduced in the presence of preferred carbon sources, commonly in response to cellular cyclic AMP levels (Deutscher, 2008; Gorke and Stulke, 2008). Consequently, the presence of additional readily degradable carbon in the unacclimated sludge may have caused a temporary reduction or inhibition of dehalogenase gene expression prior to αHA degradation. However, given that the length of the lag phase prior to TCA dechlorination was negatively correlated with the inoculum concentration, and that the longest (secondary) lag phases were observed at the lowest inoculum concentrations for both 2MCPA and TCA, this explanation seems unlikely. Indeed, the presence of additional carbon sources were apparently necessary for TCA degradation via cometabolism (Arp et al., 2001), which would explain why the TCAs RBTs conducted at lower inoculum concentrations failed to reach the RBT pass level (Chapter 5). Under current RBT protocols, the inoculum concentration is limited by the use of non-specific detection methods for detection of substrate mineralisation, which would preclude the use of the higher inoculum concentrations (Chapter 5),

despite the fact that TCA was observed to be readily degradable under these conditions.

The toxicity of the test compound could also conceivably have delayed the community acclimation in the model RBTs. Some αHA, especially MCA and DCA, are known to be toxic to bacteria, including known dehalogenating bacteria (Weightman et al., 1985). However, αHA substrates in this study were used at concentrations far lower than those previously reportedly causing toxic effects.

7.4 Enrichment of Xenobiotic Degrading Bacteria In Biodegradation Tests

Whilst each of the mechanisms discussed above may have contributed to community acclimation prior to detectable substrate degradation in the model RBTs, biodegradation will almost inevitably require a period for the enrichment of a small population of degrading organisms to a level where biodegradation is detectable (Spain et al., 1980; Ventullo and Larson, 1986; Wiggins et al., 1987; van Ginkel et al., 1995; Ingerslev et al., 2000). In this study, the enrichment of degrading organisms during RBTs was monitored using the 16S rRNA gene for phylogenetic identification and dehalogenase genes reflecting the specific dehalogenating community. RBTs failing to meet the pass level during this study were always associated with delayed enrichment of dehalogenase containing organisms, and in one instance, the total absence of any dehalogenase containing organisms during the latter stages of the test (test 0.3C, Chapter 4).

The time taken to enrich dehalogenating organisms was related to the inoculum concentration used during the RBT, with lower inoculum concentrations demonstrably influencing the levels of specific degraders within the starting community, as seen by the presence or absence of *dehI* and *dehII* PCR products, thus increasing the time taken for their enrichment. Previous studies have shown that reducing inoculum concentration and diversity has greater impact on those processes carried out by minor constituents of the community, such as nitrification and methanogenesis (Toyota et al., 1999; Wu et al., 2002) and also that smaller, less diverse communities are less able to deal with external stress (Franklin and Mills, 2006), such as the introduction of a xenobiotic to RBTs in this study. Reducing the

inoculum size specifically effected the enrichment of dehalogenating organisms containing *dehI* genes, which are essential for complete dechlorination of 2MCPA, and seemingly also for TCA dechlorination, and have been shown in this and previous studies to be far less abundant and diverse in the environment than are *dehII* genes (Hill et al., 1999; Marchesi and Weightman, 2003b). Therefore, RBT failures at lower inoculum concentrations likely represent a false negative, due to a lack of specific degrading organisms in the original inoculum.

7.5 Towards a Better RBT System

The RBT serves as the first test in a tiered system of biodegradation tests designed to determine the environmental fate of chemical substances. As a first stage in the assessment of any chemicals biodegradability, the RBTs are designed to be rapid, simple and cost effective methods to assess biodegradability, and historically their value has been in identifying more easily biodegradable chemicals, not differentiating the more persistent ones (ECETOC, 2007). Therefore, many of the test conditions used are compromises included for practical reasons with little scientific justification, and do not accurately reflect any specific environment or ecosystem (Blok et al., 1985). The principal reason for most of these compromises stems from the use of non-specific detection methods for monitoring substrate degradation, which necessitate the use of both relatively high substrate concentrations and low inoculum concentrations. Given previously reported concerns regarding the use of environmentally relevant substrate concentrations for RBTs (Ingerslev et al., 1998; Ahtiainen et al., 2003), it is perhaps surprising that environmentally relevant (i.e. higher) inoculum concentrations have not warranted more consideration.

The concentrations of activated sludge found in the aeration basin of sewage treatment plants used as the source of inoculum for this study ranged from 2.7 – 4.3 g/l, 100-fold higher than the standard sludge concentration for the RBT. This study demonstrated that lower inoculum concentrations used during model RBTs consistently gave more variable RBT results, and a relatively higher proportion of RBT fail results. The variability and test failure was linked to the presence of and functional redundancy of relevant degrading (dehalogenase containing) organisms within the test system. As such, higher inoculum concentrations increased the number and diversity of specific degrading organisms within the test system, thus improving

chances of obtaining a positive RBT result, and reducing the test's variability. In a study examining more generalised community functionality, as indicated by substrate utilisation in Biolog GN microplates, Franklin et al. (2001) also showed that a reduction in community function coincided with a reduction in diversity. Curtis et al. (2002) showed that the bacterial diversity of a community may be estimated from just two parameters: (a) the abundance of the most dominant member of the community; and (b) the total number of members in that community – assuming a log-normal species abundance curve. Using such models, it was demonstrated that larger populations contain a greater diversity than smaller populations (Curtis et al., 2002; Curtis and Sloan, 2004), and therefore, as discussed above, carry greater functional redundancy. This would imply that the larger the inoculum size the higher the probability of achieving a positive result from an RBT.

As shown in Chapter 3, the use of a standardised inoculum based on lyophilised activated sludge showed some potential for future use as an activated sludge surrogate in the RBT, and could presumably alleviate some of the variability associated with RBTs by providing a consistent starting inoculum for each test. However, future work will be required in order to establish the stability and applicability of such an inoculum for use in the RBT.

As current RBT methods are incompatible with the use of higher inoculum concentrations, several studies have called for the use of pre-adapted inocula in order to increasing the number of specific degrading organisms present in the inoculum (van Ginkel et al., 1995; Thouand et al., 1996; Toräng and Nyholm, 2005). Pre-adapted inocula could then be used at standard concentrations for RBTs. However, this study has shown that the standard 30 mg/l RBT inoculum may contain insufficient additional readily degradable carbon to allow cometabolism to take place, which is another of the RBT conditions that does not accurately reflect the situation in the environment, and another reason for the incorporation of higher, more realistic inoculum concentrations for biodegradation testing.

The microbial composition of activated sludges varies significantly both within and between different waste water treatment plants (Forney et al., 2001), which is thought to be the principal reason for variable biodegradation testing results observed

previously (Forney et al., 2001; Ahtiainen et al., 2003). Therefore, it is likely that the RBTs, and all biodegradation tests utilising environmental samples as inocula, will always contain an element of unpredictability in their results (Blok et al., 1985). However, this study suggests that using higher, more environmentally relevant, inoculum concentrations would reduce the number of false negative classifications of chemicals in the RBT. If, as is currently the case, the use of higher inoculum concentrations is not acceptable to regulatory authoroties, then the use of pre-adapted inocula as a means of increasing the number of relevant degrading organisms should be considered for use in RBTs.

7.6 Dehalogenases and Dehalogenase Producing Bacteria

The αHAs used during this study were ranked, in order of greatest recalcitrance, TCA>2MCPA>DCA, which is consistent with previous investigations (Weightman et al., 1992; Ellis et al., 2001). 2MCPA is given an intermediate ranking as its degradability was very highly influenced by the inoculum size. Despite being chemically very similar these compounds posed markedly different challenges and selection pressures to the bacterial community. Exposure to DCA and TCA resulted in the enrichment of *dehII* and *dehI* genes, respectively, but 2MCPA consistently resulted in enrichment of both *dehI* and *dehII* genes. 2MCPA dechlorination was commonly accomplished in two phases, an observation that has not been reported previously in the literature. Biphasic dechlorination was attributed to the separate enrichment of DehIIs, followed by DehIs which were supposed to degrade L-2MCPA and D-2MCPA respectively, and was probably a result of the abundance and diversity of these two gene families in the unacclimated inoculum. It was shown that the activated sludge inoculum used carried a strong functional redundancy for *dehII* genes making it better able to adapt following a reduction in size, which was not the case with respect to *dehI* genes.

Figure 7.1 shows the phylogenetic relationship between all *dehI* genes amplified in this study, all of which were assigned to either subgroups B or C, as defined previously (Hill et al., 1999). Cluster *dehI* M1 (Figure 7.1) represents a dehalogenase with activity against both 2MCPA and TCA and consists of clusters I-1 and I-2 (Chapter 3), *dehI* cluster 3 (Chapter 4) and *dehI* cluster 2 (Chapter 5), all of which shared > 97% nucleotide sequence identity, and were associated with members of the

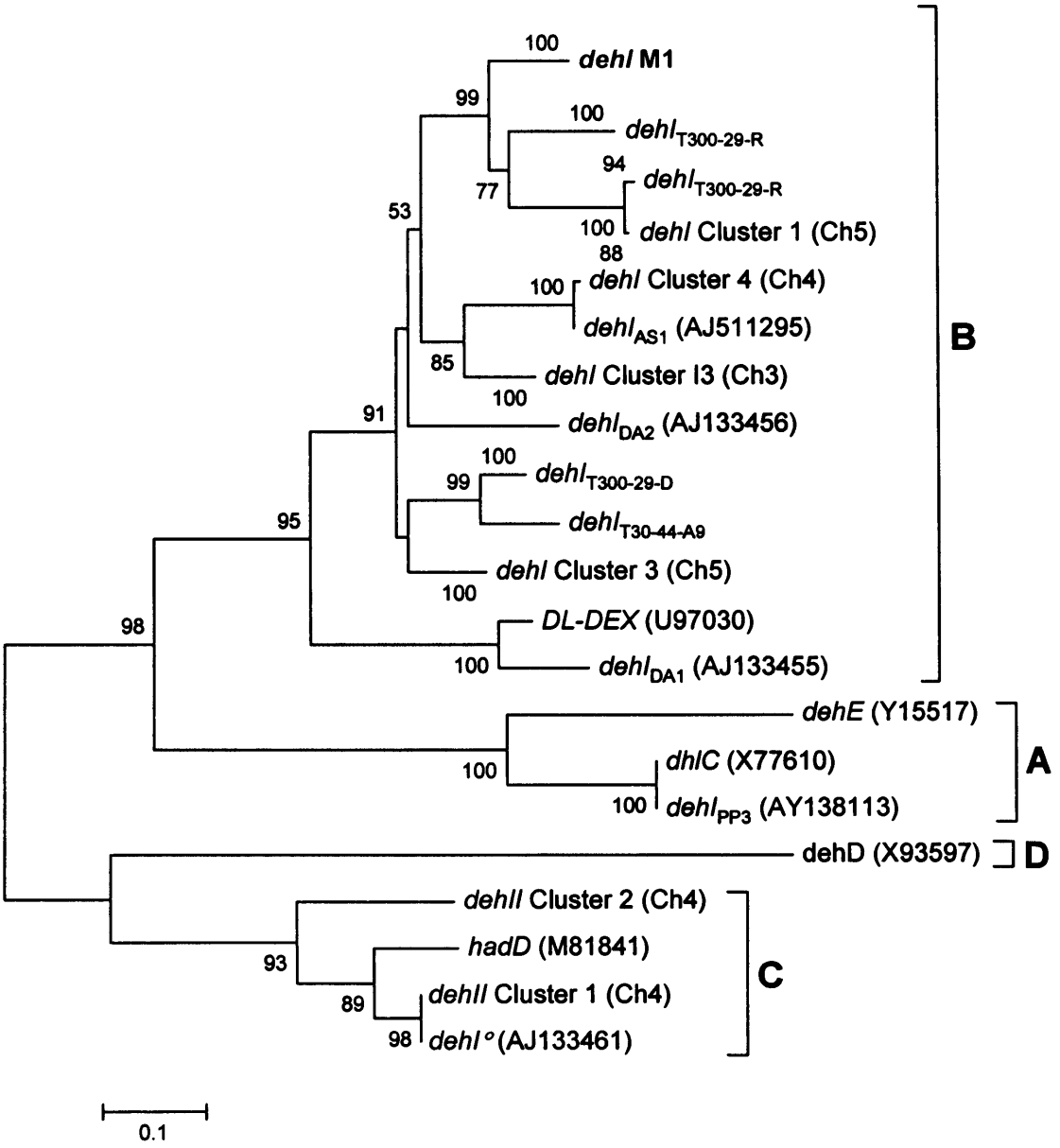

Figure 7.1 Dendrogram illustrating the phylogenetic relationship between *dehI* genes and their transcripts amplified from this study and references from the database. Samples labelled in red are specifically referred to in the text. The tree was constructed from a final dataset of 221 nucleotides using the Neighbor-Joining method (Saitou and Nei, 1987) and the Jukes-Cantor algorithm (Jukes and Cantor, 1969). Bootstrap values > 50, calculated from 1000 replicates, are shown as percentages at the nodes. Subgroups were defined previously by Hill et al. (1999). The scale bar represents the number of base substitutions per site. Accession numbers are included in the parenthesis. For clone identification, see Chapters 3, 4 and 5.

Alphaproteobacteria (*Xanthobacter* sp. in Chapter 3, *Starkeya* sp. in Chapter 4 and a *Bradyrhizobium* sp. in Chapter 5). Similarly, cluster *dehII* M2 (Figure 7.2C), which consisted of cluster II10 (Chapter 3) and *dehII* cluster 7 (Chapter 4), was also associated with two *Alphaproteobacteria* (*Xanthobacter* sp. in Chapter 3, *Starkeya* sp. in Chapter 4). Previous studies have shown little congruence between phylogenies based on the 16S rRNA gene and dehalogenase genes, probably due to the HGT of dehalogenase genes among different bacteria (Marchesi and Weightman, 2003b), although cluster *dehI* M1 and *dehII* M2 represent dehalogenases consistently associated with the *Alphaproteobacteria*.

The *dehII* genes amplified in this study (Figure 7.2) were considerably more diverse than the *dehI* genes. The diversity of the *dehII* genes assigned to subgroups A and C (48% and 53% nucleotide sequence identity, respectively) was such that these subgroups no longer conform to the original parameters (where a subgroup was defined as sequences sharing > 55% nucleotide sequence identity) designated by Hill et al. (1999). Additionally, two groups of dehalogenases from unacclimated inocula (U1 and U2, Figure 7.2) and one group of three clones from 2MCPA RBTs described in Chapters 3 and 4, consistently formed groups outside the recognised *dehII* subgroups, and did not contain any *dehII* genes from cultivated organisms. Cluster U1 contained the clone $dehII_{ASgDNA\text{-}1}$, which was identified previously in activated sludge and tentatively described as a novel subgroup (Marchesi and Weightman, 2003b). None of the genes from groups U1, U2 or U3 were transcribed at a detectable level at any point in the RBTs, although they did contain the conserved amino acid residues essential for catalysis (Kurihara et al., 1995; Hisano et al., 1996) and those forming the hydrophobic pocket of the enzyme (Li et al., 1998) in their derived amino acid sequences. However, it should be noted that the *dehII* PCR products amplified in this study represent only a small portion of the whole gene, containing only 4 from 16 of the known essential amino acid residues in DehIIs. Given the close evolutionary relationship between DehII enzymes and other members of the haloacid dehalogenase (HAD) superfamily of hydrolases, and the highly degenerate nature of the *dehII* PCR primers (Hill et al., 1999), these *dehII* genes can not be definitively classified until a cultivated organism containing a representative is obtained. Alternatively, a representative could be sought using metagenomic approaches, using DNA hybridisation, and/or PCR, and/or functional (dehalogenase activity) screening of

large insert metagenomic libraries. Interestingly, *dehII* M1 consisted of *dehII* cluster 2 (Chapter 4) and 15 identical clones from unacclimated sludge in TCA RBTs (Chapter 5). This suggests that despite its presumed activity against 2MCPA (Chapter 4), this dehalogenase had no activity against TCA.

Rhodococcus sp. strain R1, isolated in this study (Chapter 6) represents the only *Actinomycete* reported to date that is capable of growing using αHAs as sole carbon and energy sources. Whilst the dehalogenase identified in this strain, designated DehR1, was similar in activity to previously reported group I αHA dehalogenases (showing activity with L- and D-2MCPA), no αHA dehalogenase gene was amplified from this strain with the Hill et al. (1999) primers. Therefore, this may represent a divergent member of one of the two currently identified dehalogenase gene families, or an entirely novel αHA dehalogenase. In a recent study, Chiba et al. (2009) identified a *Bacillus* strain from marine sediment with activity against bromoacetic acid. The dehalogenase purified from this strain also showed activity against both D- and L-2MCPA, although no product was amplified from extracted DNA using αHA dehalogenase PCR primers (Hill et al., 1999). This suggests the possible existence of a novel dehalogenase lineage associated with Gram positive bacteria. *Rhodococcus* sp. strain R1 can now be used to identify the gene(s) encoding these dehalogenase(s), so as to establish their phylogenetic relationship, if any, to previously characterise αHA dehalogenases, and also to elucidate their mechanisms of action.

7.7 Future Perspectives

Whilst this study has provided valuable insights into the processes contributing to the acclimation of bacterial communities to degrade halogenated xenobiotics, recent developments in cultivation-independent analysis show promise to improve further our understanding of the processes involved in microbial community adaptation to environmental challenges. Next generation sequencing (NGS) techniques are providing sequence data on previously unimaginable scales, aiding in our understanding of community structure and function by identifying novel gene clusters (Demaneche et al., 2009) or characterising catabolic gene diversity in previously unexplored environments; e.g. the phosphonoacetate hydrolase (*phnA*) genes in temperate coastal waters (Gilbert et al., 2009). NGS could provide a detailed picture

of the native (unaclimated) dehalogenase diversity present in an environment, which would clarify the currently blurred boundaries between the group II αHA dehalogenases and the broader HAD super family of hydrolases, and could also be utilised to identify dehalogenase gene clusters, in order to better understand the regulation of αHA catabolism. Deep sequencing of the dehalogenase gene pool would also allow an assessment of the abundance and diversity of αHA dehalogenases recently identified in *Archaea* (Rye et al., 2009). Stable isotope labelling of αHA substrates could also be used to more directly identify the active component of the community, distinguishing them from non-active background community members, as demonstrated previously using other chlorinated xenobiotics (Borodina et al., 2005).

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