CHARACTERISATION OF CHLORINATED-HYDROCARBON-DEGRADING GENES OF BACTERIA.

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As the candidate's supervisor, I have approved this thesis for submission.

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ABSTRACT

1,2-dichloroethane (DCA) is one of the most widely used and produced chemicals of the modern world. It is used as a metal degreaser, solvent, chemical intermediate as well as a fuel additive. This carcinogen is toxic to both terrestrial and aquatic ecosystems and accidental spills and poor handling has resulted in contamination of the environment. Thus far several bacteria in the Northern hemisphere have been identified that are capable of utilizing this compound as a sole carbon and energy source. This report focuses on the isolation and characterization of bacterial isolates from the Southern hemisphere that are capable of degrading DCA as well as the global distribution of the DCA catabolic route. Samples obtained from waste water treatment plants were batch cultured in minimal medium containing DCA and repeatedly sub-cultured every five days over a 25 day period. A halogen release assay was performed in order to determine whether individual isolates possessed dehalogenase activity. Confirmation of DCA utilization by bacterial isolates positive for dehalogenase activity was done by sub-culturing back into minimal medium containing DCA. Enzyme activities were confirmed with cell free extracts using all of the intermediates in the proposed DCA degradative pathway and compared to a known DCA degrading microorganism. Biochemical tests and 16SrDNA sequencing indicated that all the South African isolates belonged to the genus *Ancylobacter* and were different from each other. Based on enzyme activities, it was found that the South African isolates may possess a similar degradative route as other DCA degrading microorganisms. Primers based on genes involved in DCA degradation were synthesized

and PCR analysis was performed. It was found that all isolates possessed an identical hydrolytic dehalogenase gene whereas the other genes in the pathway could not be PCR amplified. Southern hybridization using probes based on known genes indicated that some of the isolates had homologous genes. Pulsed field gel electrophoresis (PFGE) and random amplified polymorphic DNA (RAPD) analysis indicated that the five South African isolates of *Ancylobacter aquaticus* are distinguishable from each other. This study is the first report indicating that microbes from different geographical locations use similar metabolic routes for DCA degradation. The first gene of the pathway (*dhlA*) has undergone global distribution which may be due to widespread environmental contamination.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

Halogenated compounds are an extremely important and diverse class of environmental chemicals. Most of these compounds are used as intermediates in the chemical industry, as solvents and in some cases as pesticides (Leisinger, 1996). A large proportion of terrestrial and aquatic contamination can be attributed to accidental leakage during production, application or transport. These compounds are extremely hazardous to humans and wildlife. In recent times emission of halogenated compounds has decreased significantly although there are still large amounts being released into the environment (de Wildeman and Verstraete, 2003).

Microbiological research on the biodegradation of halogenated compounds has mainly focused on the physiological processes responsible for their mineralization and on the enzymes involved in the cleavage of the carbon-halogen bond. The characterization of dehalogenating organisms is important for their industrial application, because their physiological properties and substrate range will determine the process conditions that should be used and the range of transformations that can be obtained in practical treatment systems (Janssen *et al.*, 2001).

1.1 OCCURENCE OF HALOGENATED COMPOUNDS

Halogenated compounds are mainly used as degreasing agents, detergents, paint removers, solvents and chemical intermediates in the production of a whole range of products. Chlorinated ethanes can be used as intermediates for the production of chloroethenes, explosives and dyes and in the rubber-vulcanising processes (de Wildeman and Verstraete, 2003). Among all of the halogenated compounds, 1,1,1-trichloro- and 1,2-dichloroethane (DCA) are produced in largest quantities. Production figures for 2006 indicate that Europe produced 1.441 million tons, Asia produced 3.514 million tons and the United States of America produced 9.732 million tons of DCA (Anonymous, 2008).

Leaks, poor handling and improper disposal have resulted in C2 to C4 compounds being the most frequently encountered contaminants in soil and ground-water. It has been observed that the lower chlorinated (C2 to C4) compounds accumulate in environments where reductive conditions prevail and under these conditions their half life generally exceeds several decades (de Wildeman and Verstraete, 2003). These compounds occupy the top rankings of the American and European priority list of environmental pollutants (Hughes *et al*., 1994). It has been found that exposure to chlorinated alkanes can be hazardous to humans and wildlife, with some molecules resulting in cell damage, cancer and death (de Wildeman and Verstraete, 2003). The toxic effects of chlorinated alkanes in mammals mainly occur during metabolic activity when these compounds are transformed into more toxic products. A good example is DCA which is oxidatively

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converted to chloroacetaldehyde which has known carcinogenic effects (Premaratne *et al.*, 1995).

The properties and solubility of chloroalkanes influence the probability of encountering these pollutants in the environment especially groundwater. Some of the C2 to C4 chloroalkanes may have relatively high solublities (200 – 8000mg/L) and their high resistance to reduction reactions influences their prevalence in aquifers which are important reservoirs for these pollutants (de Wildeman and Verstraete, 2003). Most currently encountered halogenated compounds are of xenobiotic origin but there is also an abundance of halogenated organic compounds that are naturally produced. There are more than 2000 halogenated chemicals known to be released into the biosphere by various marine organisms, higher plants and ferns, insects, bacteria, fungi and mammals (Gribble, 1994).

1.2   1,2-DICHLOROETHANE (DCA)

1.2.1   Properties

1,2-Dichloroethane also called 1,2-ethylene dichloride, dichloroethylene or ethylene dichloride is a manufactured chemical that is not found naturally in the environment. Visibly it is a clear, colorless, oily liquid. It is highly flammable and has a pleasant, chloroform-like odour. It is generally soluble in most organic solvents and sparingly (8.69×10^3mg/L at 20°C) soluble in water (Agency for Toxic Substances and Disease

Registry, 2001). Toxic fumes of hydrochloric acid are produced whenever DCA is heated to decomposition (Agency for Toxic Substances and Disease Registry, 2001; National Toxicology Program, 2005).

1.2.2 Use

In recent times the use of DCA as a solvent and degreaser has been replaced by less toxic compounds. It was once used as a solvent for processing pharmaceutical products and as an emulsifier of fats, oils, waxes; gums, resins and particularly for rubber and also incorporated into paint, varnish and finish removers. In agriculture, it was used as an insect fumigant for stored grains and mushroom houses and as a soil fumigant in peach and apple orchards and as an extractant in certain food processes. Household products containing DCA included cleaners for upholstery and carpets and industrially it was used as a solvent in textile cleaning and metal degreasing. In the petro-chemical industry it was used as a lead scavenger in antiknock gasoline. It is also used industrially as a starting material for chlorinated solvents such as vinylidene chloride, a dispersant for plastics and elastomers such as synthetic rubber. DCA has also been used as a general anesthetic instead of chloroform, especially in ophthalmic surgery. At present it is used primarily to produce vinyl chloride which is an intermediate in the production of poly vinyl chloride or PVC as it is commonly referred to as (Agency for Toxic Substances and Disease Registry, 2001; National Toxicology Program, 2005).

1.2.3   Health effects

Humans are generally exposed to DCA by inhalation, ingestion or dermal contact. Breathing high levels of DCA may result in many harmful effects to people. Damage to the heart, central nervous system, liver, kidneys and lungs has been seen in people who accidentally ingested high doses of this chemical. The greatest source of exposure to DCA for most people is inhalation of the compound in contaminated air. Releases of this compound to the environment may result from the manufacture, use, storage, distribution and disposal of DCA. Currently, occupational exposure is chiefly to workers involved in the production of vinyl chloride (Williams *et al.*, 2001; National Toxicology Program, 2005).

DCA has been detected up to concentrations of 0.005mM not only in surface water, drinking water and groundwater but also in ambient urban and rural air and indoor samples of residences located near hazardous waste disposal sites (National Toxicology Program, 2005). DCA is primarily released to the environment through air. DCA can stay in the air for more than five months before it is broken down in air by reacting with other compounds formed by sunlight. DCA released into rivers and lakes breaks down very slowly and most of it will evaporate into the air. In soil DCA will either evaporate into the air or travel down through the soil and enter ground water. Exposure of the general population to DCA is generally by breathing air or drinking water that contains DCA. People living near uncontrolled hazardous waste sites or live or work near a factory where DCA is used may also be exposed to higher than usual levels of DCA

(Agency for Toxic Substances and Disease Registry, 2001; National Toxicology Program, 2005).

Some of the common diseases reported in humans ingesting or inhaling large amounts of DCA include nervous system disorders, liver and kidney diseases and lung damage. Laboratory animals exposed to large amounts of DCA by inhalation or ingestion have shown nervous system disorders and liver, kidney and lung damage as well as damage to the immune system. Animals ingesting low doses of DCA for a long time have shown kidney damage. Although human studies examining whether DCA can cause cancer have been considered inadequate, in animals, increases in the occurrence of stomach, mammary gland, liver, lung and endometrium cancers have been seen following inhalation, oral and dermal exposure. It is not known whether DCA will result in birth defects or other developmental effects in people and studies in animals suggest that DCA does not produce birth defects. It can also be considered likely that health effects seen in children exposed to high levels of DCA will be similar to the effects seen in adults. At present the Environmental Protection Agency (EPA) and the International Agency for Cancer Research (IARC) have deemed DCA to be a probable human carcinogen (Agency for Toxic Substances and Disease Registry, 2001; Williams *et al*., 2001; National Toxicology Program, 2005).

1.3   BACTERIAL DEGRADATION OF XENOBIOTIC COMPOUNDS

Synthetic chemicals are generally resistant to biodegradation mainly due to the lack of enzymes that can carry out critical steps in a catabolic pathway. Low molecular weight halogenated compounds are especially recalcitrant.   Many of these compounds are relatively water soluble and are bio-available to microorganisms to be converted via short metabolic routes to intermediates that support cellular growth under aerobic conditions. However, no microorganisms have been found that oxidatively degrade and use as a carbon source important environmental chemicals such as chloroform, trichloroethylene, 1,1,1-trichloroethane, 1,2-dichloropropane and 1,2,3-trichloropropane. There have been many unsuccessful attempts to obtain enrichments or pure cultures that aerobically grow on these chemicals (Janssen *et al*., 2005). Other compounds such as chloroacetate, 2-chloropropionate and 1-chlorobutane are more readily biodegradable and degrading organisms can be easily isolated from soil samples (Leisinger, 1996). Some compounds such as DCA, dichloromethane and the nematocides 1,2-dibromoethane and 1,3-dichloropropene are not readily degraded by microorganisms.   Degradative microorganism can only be isolated after a prolonged adaptation or if a suitable inoculum is used in which the catabolic activity that is searched for is already enriched due to pre-exposure to halogenated chemicals in the environment (Janssen *et al*., 2005).

In any particular degradation route a key step would be dehalogenation to remove one or more halogen atoms. At present a wide range of dehalogenase enzymes have been characterized that catalyze the cleavage of carbon-halogen bonds in both aliphatic as well

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as aromatic compounds. A variety of reaction types are catalyzed by these enzymes and they may or may not require co-factors for activity.

1.4   BIOLOGICAL DEHALOGENATION REACTIONS

With the isolation and characterization of microorganisms capable of degrading halogenated compounds, a number of dehalogenases and dehalogenation mechanisms became better understood.

1.4.1   Hydrolytic dehalogenation

The first purified bacterial dehalogenase catalysed the dechlorination of chloroacetate and 2-chloropropionic acid (Jensen, 1960). It was later found that similar 2-haloacid dehalogenases could be isolated from many different bacteria such as *Moraxella* sp., *Pseudomonas* sp. *Ancylobacter aquaticus* AD25 and AD27 (van den Wijngaard *et al*., 1992) and *Xanthobacter autotrophicus* GJ10 (van der Ploeg *et al*., 1991). These enzymes catalyse a substitution of the halogen with water as a nucleophile resulting in the formation of an alcohol (Fig. 1) and no other co-factor is required (van Pee and Unversucht, 2003).
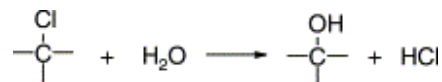
Fig. 1.1:   Hydrolytic dehalogenation. In hydrolytic dehalogenation reactions the halogen is displaced by water in a nucleophilic replacement reaction (van Pee and Unversucht, 2003).

One of the best studied hydrolytic dehalogenases is the haloalkane dehalogenase from the DCA degrading bacterium, *X. autotrophicus* GJ10. Studies using X-ray crystallographic analysis (Verschueren *et al*., 1993) and site directed mutagenesis (Pries *et al*., 1994) have elucidated the reaction mechanism of this enzyme. It belongs to the α/β hydrolase fold family and contains a catalytic triad consisting of one histidine and two aspartate residues (Fig. 1.2) which are involved in the nucleophilic substitution of the halide ion by water. Removal of the halogen as a halide ion is additionally facilitated by two tryptophan residues (Kennes *et al*., 1995).

Haloalkane dehalogenases function in a two step catalytic mechanism similar to lipases, esterases, carboxypeptidases and acetylcholinesterases except that the nucleophile is an aspartate instead of a serine/cysteine residue which attacks the halogen-bearing sp^3-hybridised carbon atom in an S_N2-type substitution mechanism. The covalently bound ester intermediate that is formed is easily hydrolysed by attack of a histidine-activated water molecule on the Cγ atom of the aspartate. The substitution of the serine/cysteine of an α/β-hydrolase by an aspartate confers on the enzyme an essential prerequisite to hydrolyse carbon-halogen bonds in haloalkanes which additionally contain a halide binding site to facilitate the dehalogenation reaction (Hunkeler and Aravena, 2000; De Jong and Dijkstra, 2003).
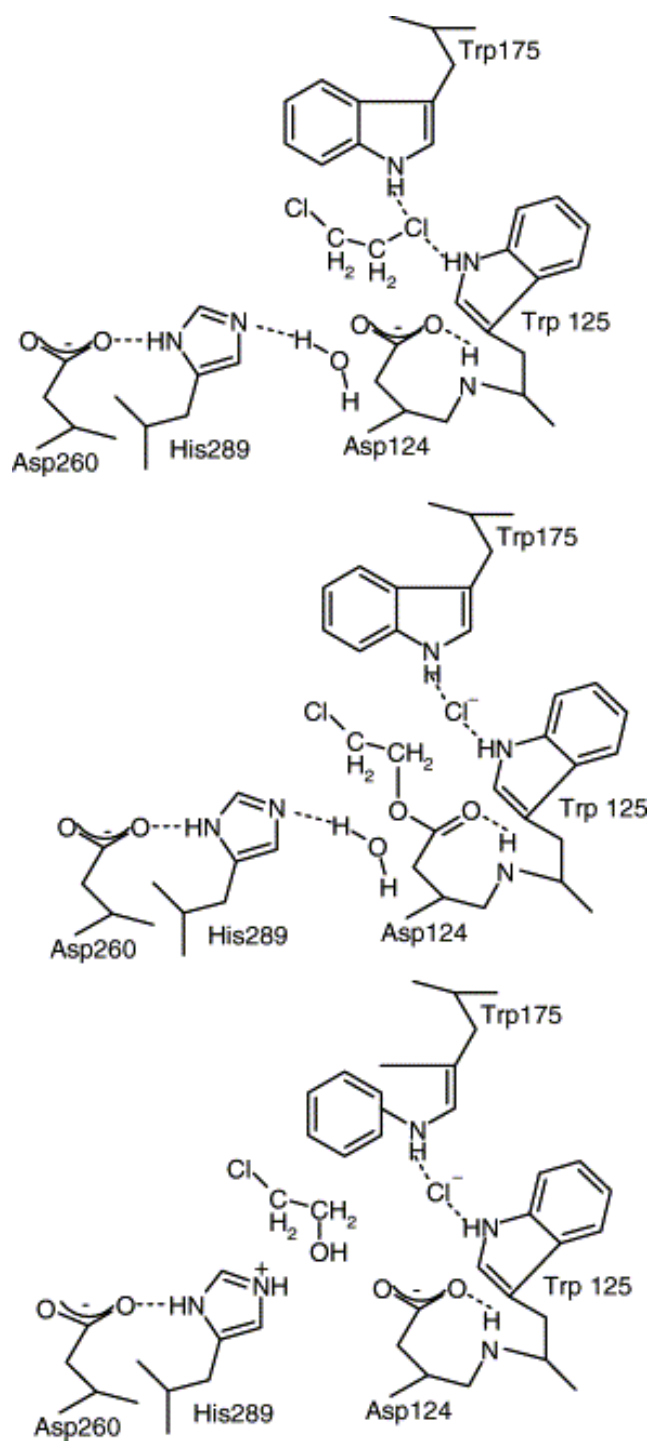
Fig. 1.2:   Mechanism of haloalkane dehalogenase.   Two trytophan residues are involved in
removing the halogen atom (van Pee and Unversucht, 2003).

Biological dehalogenation reactions are also carried out by haloacid dehalogenases which catalyse the hydrolysis of α-halogenated carboxylic acids, such as 2-chloroacetate, which is an intermediate in the degradation of 1,2-dichloroethane. The members of the haloacid dehalogenase (HAD) superfamily include magnesium-dependant phosphatases and P-type ATPases. These enzymes are dimers with two or three domains per sub-unit: a core domain with a Rossmann-fold-like six stranded parallel β sheet flanked by five α helices which is completely different from the α/β-hydrolase fold of the haloalkane dehalogenases. The halide binding site is different and utilizes aspartate-based catalytic mechanism that proceeds via a covalent intermediate but there is no histidine to activate the water molecule. It is not known how the water molecule is activated but it has been suggested another aspartate in the active site fulfills this function (De Jong and Dijkstra, 2003, Burrows *et al*., 2006).

The 4-chlorobenzoyl-CoA dehalogenase from *Pseudomonas* sp strain CBS-3 is a heterodimer in which each subunit folds into two domains. The structure of the enzyme consists of an N-terminal domain containing a ten-stranded β sheet, forming two nearly perpendicular layers, which are flanked by α helices while the C-terminal domain is composed of three amphiphilic α helices and is primarily involved in trimerisation. The mechanism by which a halogen is displaced from the aromatic ring of 4-chlorobenzoate, a degradation product of PCBs (polychlorinated biphenyl) proceeds via a covalent aspartyl intermediate. The substrate is first ligated to CoA (coenzymeA) by 4-chlorobenzoate CoA ligase which is then bound by 4-chlorobenzoyl-CoA dehalogenase with the enolate anion of the thioester link of the substrate stabilized by two backbone

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amide groups. The partially induced positive charge on the halogen-bearing carbon atom makes it susceptible to nucleophilic attack by the aspartate. Restoration of the aromatic ring system results in the chloride being expelled producing a second arylated enzyme intermediate that is subsequently hydrolysed by attack on the Cγ atom of the nucleophilic aspartate by a histidine activated water molecule yielding 4-hydroxybenzoyl-CoA (De Jong and Dijkstra, 2003).

1.4.2 Thiolytic dehalogenation

In methylotrophic bacteria such as *Methylophilus* sp., *Methylobacterium* sp. and *Hyphomicrobium* sp., the production of an inducible glutathione S-transferase when grown with dichloromethane as a growth substrate catalyses the formation of an unstable *S*-chloromethyl glutathione intermediate (Fig. 1.3) which is then converted to glutathione, chloride and formaldehyde (Leisinger *et al*., 1994; Marsh and Ferguson, 1997; Gisi *et al*., 1998).
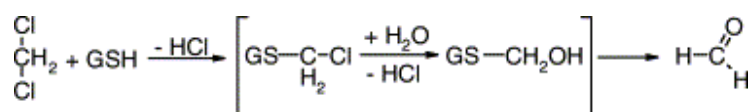
Fig. 1.3:   Thiolytic dehalogenation of dichloromethane catalysed by a glutathione transferase (van Pee and Unversucht, 2003).

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The methylotrophic bacterium *Methylophilus* sp. Strain DM11 is capable of catalysing the dechlorination of dichloromethane to formaldehyde via a highly reactive, genotoxic intermediate, *S*-(chloromethyl) glutathione (GS-CH$_2$Cl) with the aid of a glutathione (GSH)-dependent dichloromethane dehalogenase. Although this enzyme is highly efficient it is not clear how the bacterium mitigates the genotoxic effects of the heavy load of *S*-(chloromethyl) glutathione produced during the catabolism of dihalomethanes (Stourman *et al*., 2003).

1.4.3   Intramolecular substitution

Another group of enzymes called haloalcohol dehalogenases isolated from *Arthrobacter* sp. and other bacteria catalyse the intramolecular nucleophilic displacement of a halogen by a vicinal hydroxyl function in halohydrins to yield epoxides (Fig. 1.4). These enzymes are also sometimes known as halohydrin dehalogenases or halohydrin hydrogen-halide lyases (van Hylckama Vileg *et al*., 2001). These enzymes show sequence homology with the family of short-chain dehydrogenases/reductases but do not require NAD(P)+ as a co-enzyme. These enzymes have the same serine and tyrosine active site amino acid residues, but the lysine residue of the short chain dehydrogenases/reductases is replaced by an arginine residue. Haloalcohol dehalogenases are also able to catalyse the reverse reaction, the halogenation of epoxides to haloalcohols (van Pee and Unversucht, 2003).
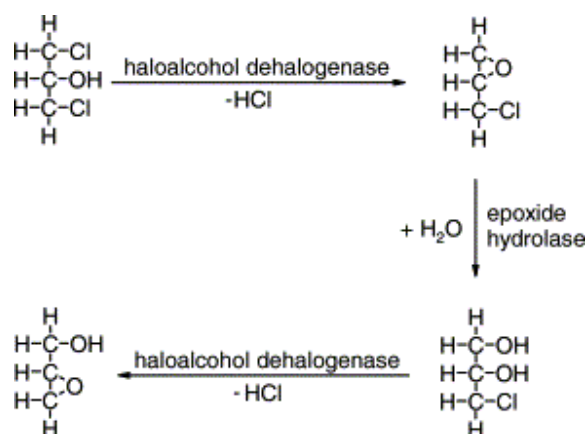
Fig. 1.4: Intramolecular nucleophilic displacement of a halogen by a vicinal hydroxyl group catalysed by haloalcohol dehalogenases. These enzymes can also catalyse the reverse reaction, the halogenation of the epoxides to haloalcohols (van Pee and Unversucht, 2003).

Thus far, six different isolated haloalcohol dehalogenases have been grouped into the three sub-types (A, B and C) on the basis of amino acid sequence similarities which also indicate that these enzymes are evolutionarily related to the large family of NAD(P)(H)-dependent short chain dehydrogenases/reductases (SDRs) which use a similar Ser-Tyr-lys/Arg catalytic triad to catalyse various alcohol-ketone conversions. The tyrosine residue of the conserved Ser-Tyr-Arg catalytic triad of the haloalcohol dehalogenase HheC from *Agrobacterium radiobacter* AD1 is in a position to activate the hydroxyl group of the haloalcohol function for nucleophilic attack on the vicinal halogen bearing carbon atom. The halide product is stabilized in a spacious halide-binding site to which the halogen atom is bound. Analysis of the structure of the C-type haloalcohol dehalogenase, HheC and the SDRs indicate that the different dehalogenase subtypes may have originated from NADH-dependent rather than NADPH-dependent SDR precursors. It has also been found that the different haloalcohol dehalogenases show marked

differences in their catalytic behaviour : HheC is highly enantioselective towards various aliphatic and aromatic substrates, whereas the A- and B- type dehalogenases have only modest enantiopreference (De Jong *et al*., 2006).

1.4.4   Dehalogenation by hydration

Two dehalogenases have been detected in a coryneform bacterium isolated with 3-chloroacrylate as the carbon source.   It has been proposed (Fig. 1.5) that the dehalogenation reactions catalysed by these enzymes proceeds by the addition of water to the double bond yielding an unstable intermediate from which malonate semialdehyde is formed (van Hylckama Vlieg *et al*., 2001; Janssen *et al*., 1994).
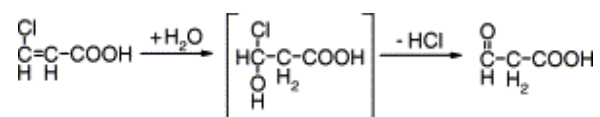
Fig. 1.5:   Dehalogenation by addition of water onto a double bond (van Pee and Unversucht, 2003).

The chloroacrylic acid dehalogenases (CaaD) displace a halogen from a sp^2-hybridised carbon atom in a manner similar to the 4-chlorobenzoyl-CoA dehalogenases. It has been found that the nematocide, 1,3-dichloropropene can be degraded by the *cis*-and *trans*-3-chloroacrylic acid dehalogenases from *Pseudomonas pavonaceae* 170 (Poelarends *et al*., 2001).   Following   conversion   of   *trans*-1,3-dichloropropene   by   a   haloalkane dehalogenase, oxidation of the *trans*-1-chloro-3-hydroxypropene product yields *trans*-3-

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chloroacrylate which is concerted into malonate semialdehyde, a chlorine ion and a proton by CaaD. Decarboxylation of malonate semialdehyde yields acetaldehyde which can be used as an alternative growth substrate (De Jong and Dijkstra, 2003).

The solved X-ray structures of native and inactivated forms of CaaD have revealed that the enzyme is a trimer of αβ-heterodimers. The enzyme fold is similar is similar to that of 4-oxalocrotonate tautomerase (4-OT), a member of the tautomerase superfamily which includes isomerases and tautomerases. Isomerases and tautomerases differ from CaaD with the catalytic Pro1 being in a hydrophobic environment whereas in CaaD the corresponding βPro1 is in a hydrophilic active site near a buried glutamate. The proline in isomerases and tautomerase functions as a general base while in CaaD it functions as a general acid (De Jong and Dijkstra, 2003).

The recently characterized tautomerase superfamily is a grouped of structurally homologous proteins that are characterized by a β-α-β building block and a catalytic amino-terminal praline (Pro-1). The signature β-α-β fold was first reported in 4-oxalocrotonate tautomerase and begins with Pro-1 at the start of a β-strand (β1) followed by an α-helix (α-1) and a 3_{10} helix which precedes a second parallel β-strand (β2). A β-hairpin which is important for hexamer formation is located close to the C-terminus where the fold ends. At present the superfamily consists five families represented by 4-OT, 5-(carboxymethyl)-2-hydroxymuconate isomerase (CHMI), macrophage inhibitory factor (MIF), *cis*-3-chloroacrylic acid dehalogenase (*cis*-CaaD) and malonate semialdehyde decarboxylase (MSAD) (Poelarends *et al*., 2008).

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1.4.5 Dehalogenation by methyl transfer

It has been found that dehalogenation by methyltransfer occurs in certain aerobic and strictly anaerobic bacteria using chloromethane or dichloromethane as the sole carbon source (Meßmer *et al.*, 1996; Vanelli *et al.*, 1998). A methyltransferase described by Coulter *et al.* (1999) contains a corrinoid-bound cobalt atom. The reaction (Fig. 1.6) occurs when cob(II)alamin in the active state is oxidized to the cob(III)alamin state by halomethane methylation and is reduced by demethylation by the methyl group acceptor ion which can be a halide or bisulfide ion.
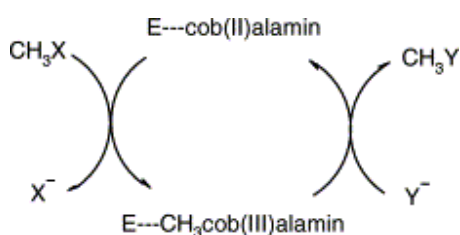
Fig. 1.6: Dehalogenation of chloromethane catalysed by a cobalamin-containing methyltransferase; CH_3X can be CH_3Cl, CH_3Br or CH_3I and Y^- can be Cl^-, Br^- or HS^- (van Pee and Unversucht, 2003).

The first step in methyl chloride metabolism by *Methylobacterium chloromethanicum* CM4 involves CmuA, a 67kDa polypepetide which has a methyltransferase domain and a corrinoid-binding domain. Transfer of the methyl group of methyl chloride to the Co atom of the enzyme-bound corrinoid group (methyltransferase I activity) occurs in the methyltransferase domain. The methyl group is then transferred onto tetrahydrofolate

(H_4F) forming methyl H_4F (methyltransferase II activity) by a second polypepetide, CmuB. The folate-linked group which is formed is then progressively oxidized to formate and finally to CO_2 to provide the reducing equivalents for biosynthesis. Carbon assimilation required for growth occurs at the level of methylene tetrahydrofolate which can feed directly into the serine cycle (McDonald *et al*., 2002)

1.4.6   Oxidative dehalogenation

These reactions can be found in the pathways for the degradation of haloaliphatic as well as haloaromatic compounds. Catalysis of these reactions are carried out by monooxygenases with a broad substrate range like the methane monooxygenase from *Methylococcus capsulatus* Bath or *Methylococcus trichosporium* (Yokota *et al*., 1986; Fox *et al*., 1990) or by dioxygenases (Fig. 1.7) like the two component 4-chlorophenylacetate 3,4-dioxygenase from *Pseudomonas* sp CBS3 (Schweizer *et al*., 1987).

The first monooxygenase mediated degradation of a chlorinated aliphatic compound (DCA) was demonstrated by Stucki *et al*. (1983). The isolated bacterium, *Pseudomonas* sp. strain DE2 was able to grow on DCA as a sole source of carbon and energy. Later *Pseudomonas* sp. strain DCA1 was isolated by Hage and Hartamns (1999) which was found to catalyze the initial step DCA metabolism using a monooxygenase. The oxidation reaction which was found to be the first step in DCA degradation by both *Pseudomonas* sp. strains DE2 and DCA1 is in contrast to the hydrolytic dehalogenation

of DCA by *X. autotrophicus* GJ10 (Janssen *et al*., 1985). Hage and Hartman (1999) observed DCA degradation in cell extracts only when NAD(P)H was added to the cell extracts suggesting that degradation was oxygen dependent requiring a monooxygenase for catalysis.

The oxygen-dependent dehalogenation of long-chain (C5 to C18) α, ω-dichloroethanes was recently observed in *Pseudomonas* sp. strain 273 (Wischnak *et al*., 1998) and hexadecane-grown cells of Rhodococcus erythropolisY2 showed oxygenase-type dehalogenation activity towards C4 to C18 α-chloroethanes (Yokota *et al*., 1986). These reactions are so common that lignin and manganese peroxidase from the lignin degrading basidiomycete *Phanerochaete chrysosporium* was also found to catalyse an oxidative dechlorination reaction of pentachlorophenol to produce tetrachloro1,4-benzoquinone (Reddy and Gold, 2000).
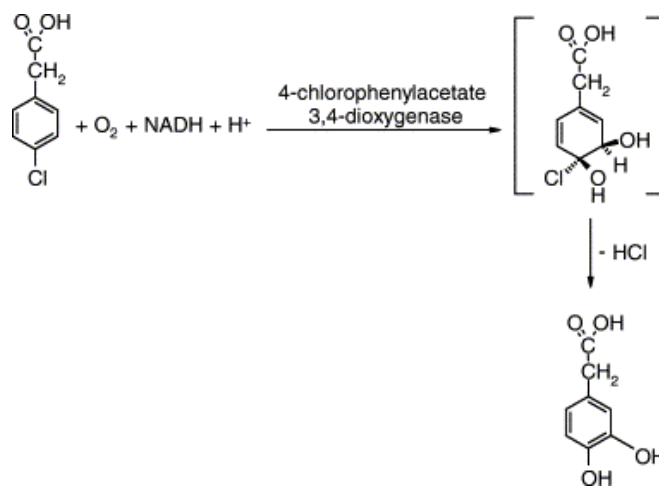
Fig. 1.7:   Oxidative dehalogenation of 4-chorophenylacetate catalysed by the two-component
            system of 4-chlorophenylacetate 3,4-dioxygenase⁻ (van Pee and Unversucht, 2003).

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1.4.7   Reductive dehalogenation

1.4.7.1   Reductive dehalogenation under aerobic conditions

Tetrachlorohydroquinone is produced by *Sphingomonas chlorophenolica* during the aerobic degradation of pentachlorophenol by a monooxygenase reaction catalyzed by pentachlorophenol hydroxylase. Tetrachlorohydroquinone dehalogenase then converts tetrachlorohydroquinone to trichlorohydroquinone (Fig. 1.8) by a reductive dehalogenation reaction (Kiefer *et al.*, 2002). It was also found that the white rot fungus *Phanerochaete chrysosporium* has a similar tetrachlorohydroquinone dehalogenase (Reddy and Gold, 2001). Like the enzyme involved in dichloromethane dehalogenation, tetrachlorohydroquinone dehalogenase is a member of the glutathione *S*-transferase family (van Pee and Unversucht, 2003).

The soil bacterium, *Sphingobium chlorophenolicum* degrades pentachlorophenol via the reductive dehalogenation of tetrachlorohydroquinone (TCHQ) to trichlorohydroquinone by a TCHQ dehalogenase which is then converted to 2,6-dichlorohydroquinone. TCHQ dehalogenase belongs to the glutathione *S*-transferase superfamily which typically catalyse the nucleophilic attack of glutathione upon an electrophilic substrate to form a glutathione conjugate. This family of enzymes are typically dimeric and contain a single glutathione binding site in each monomer whilst TCHQ dehalogenase is a monomer and uses two equivalents of glutathione to catalyse a more complex reaction (Warner and Copley, 2007).

Reductive dehalogenation of carbon tetrachloride catalysed by the lignin peroxidase from the white rot fungus *P. chrysosporium* involves the one electron oxidation of veratryl alcohol (VA) to the veratryl alcohol cation radical. The radical is then reduced by oxalacetate resulting in a one-electron oxidation of oxalacetate. Reductive dechlorination of carbon tetrachloride is then catalysed using the remaining electron in a reductive reaction (Shad *et al*., 1993).
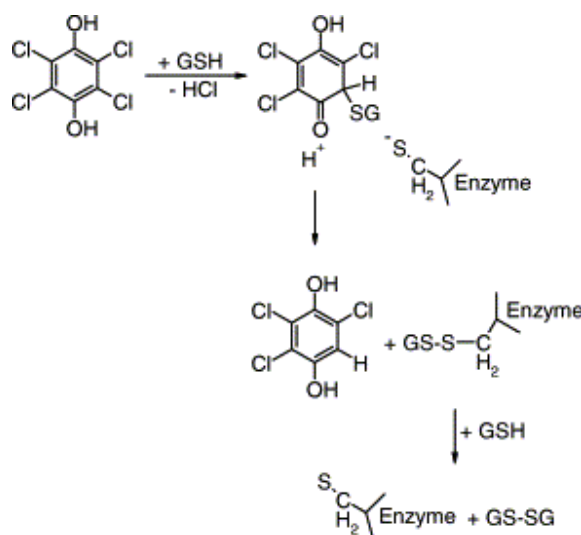
Fig. 1.8: Reductive dehalogenation of tetrachlorohydroquinone under aerobic conditions catalysed by a glutathione transferase (van Pee and Unversucht, 2003).

The reductive dehalogenation of carbon tetrachloride is a free-radical-mediated process (Fig. 1.9), initiated by the oxidation of of VA to the veratryl alcohol radical. The veratryl alcohol cation radical in turn oxidizes oxalate (excreted extracellularly by the fungus) or (ethylene diamine tetraacetic acid) EDTA to their respective radicals. Oxalate is decarboxylated with the release of CO_2^- anion radical. The EDTA radical or the carboxylate anion radical derived from either EDTA or the oxalate can reductively dehalogenate the aliphatic halocarbon (Khindaria *et al*., 1995)

A
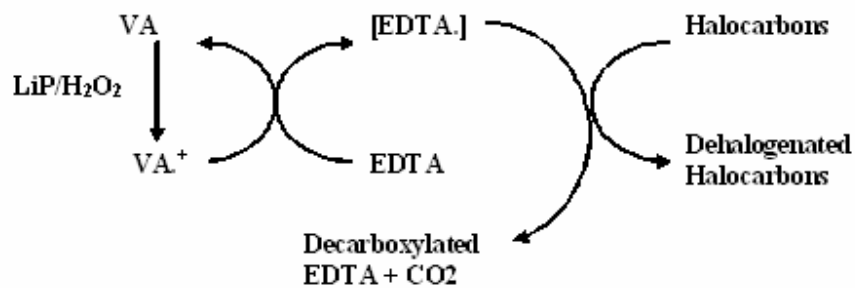
B
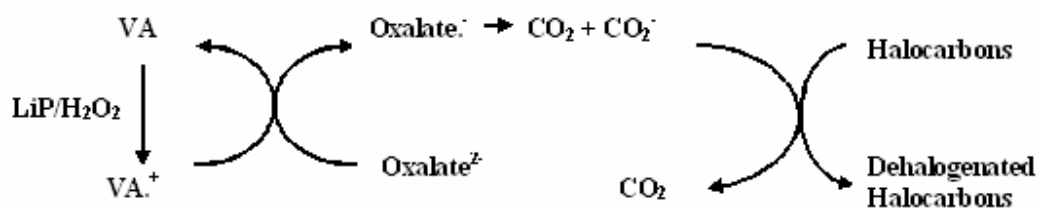
Fig. 1.9:   Proposed scheme for reductive dehalogenation of aliphatic halocarbons by lignin peroxidase using EDTA as electron donor (**A**) and using oxalate as electron donor (**B**) (Khindaria *et al*., 1995).

Halorespiration involves the coupling of reductive dehalogenation to energy metabolism. During halorespiration (Fig. 1.10) a halogenated compound like tetrachloroethene or trichloroethene serves as the terminal electron acceptor during the oxidation of an electron rich compound like hydrogen or an organic substrate (Schuhmacher *et al*., 1997 and Wohlfahrt and Diekert, 1997). Reductive dehalogenases isolated from several bacteria such as *Dehalococcoides ethenogenes* or *Desulfitobacterium dehalogenans* catalyzing these reactions contain a cobalamin cofactor and iron-sulfur clusters (Van de Pas *et al*., 1999 and Magnuson *et al*., 2000).
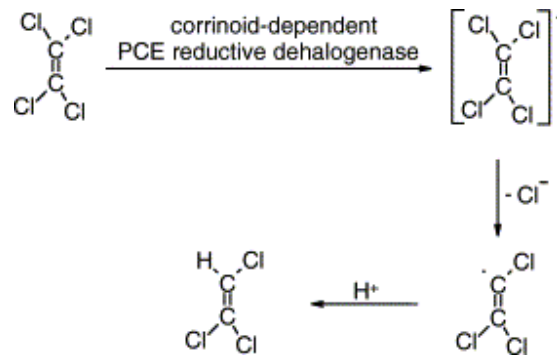
Fig. 1.10:   Corrinoid-dependent reductive dehalogenation of tetrachloroethene (PCE) under anaerobic conditions coupled to energy metabolism (halorespiration) (van Pee and Unversucht, 2003).

1.5 PHYLOGENETIC ANALYSIS OF HALOALKANE DEHALOGENASES

Structurally haloalkane dehalogenases (HLDs) belong to the α/ß-hydrolase superfamily (Nardini and Dijkstra, 1999). Analysis of the three dimensional structure of three haloalkane dehalogenases has revealed two common domains: the α/ß-hydrolase core domain (which is conserved in members of the α/ß-hydrolase superfamily) and a helical cap domain. The basic design of the α/ß-hydrolase fold is composed of an eight stranded mostly parallel β-sheet flanked by α-helices and serves as a scaffold for the main catalytic residues. The cap domain which influences the substrate specificity of these enzymes is composed of a few helices inserted into the catalytic domain, usually C-terminally to the β-strand 6 which has been found in the structure of many α/ß-hydrolases and not only hydrolytic dehalogenases. The region between the main domain and the cap domain forms the active site cavity. The catalytic pentad of residues that is essential for hydrolysis has been identified and includes Asp (nucleophile), His (base), Asp or Glu (catalytic acid) and two halide stabilizing residues, Trp and Trp or Asn (Janssen, 2004). Until recently, no classification system had been proposed for haloalkane dehalogenases that takes into consideration their phylogenetic and functional relationship although comparative biochemical analyses have been done. A recent study (Chovancova *et al*., 2007) analyzing all sequences and structures of genuine haloalkane dehalogenases and their homologs detectable by database searches revealed that the HLD family can be divided into three families denoted HLD-I, HLD-II and HLD-III of which HLD-I and HLD-III have been predicted to be sister groups. Maximum likelihood and neighbor joining analyses were conducted to infer the phylogenetic trees of the HLDs.

Experimentally characterized HLDs can be found in all three subfamilies. A subdivision of the HLD-I subfamily into two subgroups is also apparent from sequence alignment. Experimentally confirmed haloalkane dealogenases within subgroup AI include DhlA from *Xanthobacter autotrophicus* GJ10 while subgroup IB is represented by the mycobacterial dehalogenases (DmbB) from *Mycobacterium bovis* and *M. tuberculosis* and DhmA from *M. avium*. LinB from *Sphingobium japonicum*, DmbA from *M. bovis* and *M. tuberculosis*, DmsA from *M. smegmatis*, DhaA from *Rhodococcus* sp., DatA from *Agrobacterium tumefaciens*, DbjA from *Radyrhizobium japonicum*, DmlA from *Mesorhizobium loti* and surprisingly an enzyme with luciferase activity fall within the HLD-II subfamily. Although HLD-I and HLD-III may be closely related, the HLD-III subfamily currently includes two proteins that have been empirically shown to possess low dehalogenating activity i.e. DmbC from *M. bovis* and *M. tuberculosis* and DrbA from *Rhodopirellula baltica* (Chovancova *et al.*, 2007).

1.6   MECHANISM OF ACTION OF HYDROLYTIC DEHALOGENASES

The ability of haloakane dehalogenases to catalyse the conversion of an alkylhalide functionality to a alcohol group has drawn considerable attention to this group of enzymes. This prompted detailed investigations into the catalytic properties of these enzymes. Currently, X-ray structures of three different bacterial haloalkane dehalogenases have been solved and several putative dehalogenases have been cloned or detected by genome sequence analysis (Janssen, 2004).

The *X. autotrophicus* enzyme (DhlA) has become the most studied haloalkane dehalogenase and the mechanism of haloalkane dehalogenases became apparent only after the structure was solved (Verschueren *et al*., 1993). This enzyme catalyses the first step in DCA degradation (Fig. 1.11) and is composed of a α/β-hydrolase fold domain (the main domain) and smaller mostly helical cap domain (Fig. 1.12). The enzyme's active site is an occluded cavity located between both of these domains. Experimental data has suggested that catalysis is mediated by an Asp-His-Asp catalytic triad, functionally similar to the catalytic triad of serine proteases (Verschueren *et al*., 1993). The difference lies with the nucleophilic amino acid which is an aspartate rather than a serine. The reaction occurs with one of the oxygens of the aspartate carboxylate group attacking the carbon atom to which the halogen is bound replacing it by an S_N2 substitution mechanism (Fig. 1.13) (Janssen, 2004).
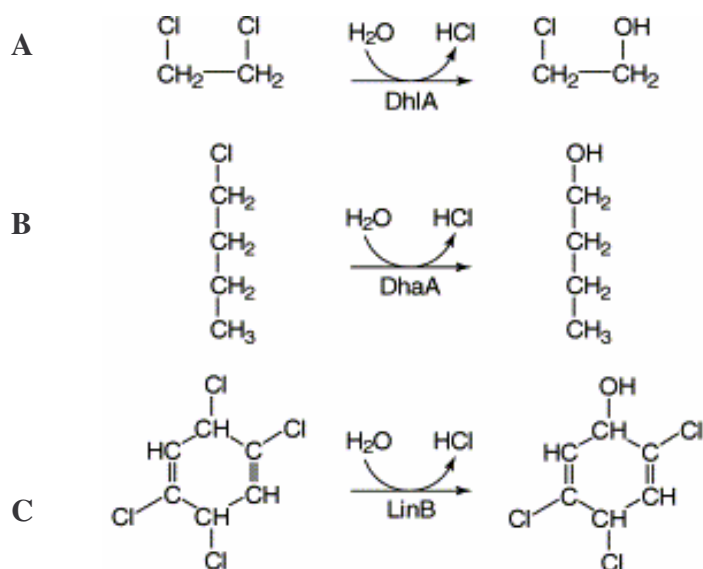
Fig. 1.11: Substrates of haloalkane dehalogenases from (**A**) *X. autotrophicus* GJ10 (DhlA), (**B**) *Rhodococcus erythropolis* NCIMB13064 (DhaA) and (**C**) *Sphingomonas paucimobilis* UT26 (LinB) (Janssen, 2004).

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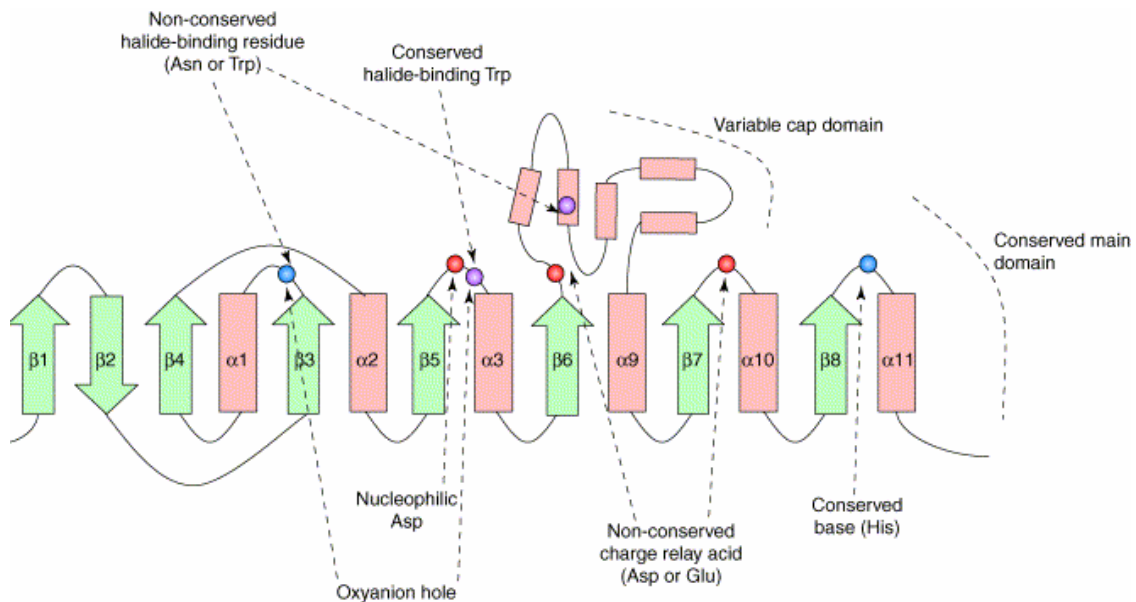
Fig. 1.12: General topology of haloalkane dehalogenases. The cap domain is mostly helical and less conserved between the different dehalogenases (Janssen, 2004).

A halide binding site is formed by two tryptophan residues, Trp125 and Trp175 which are involved in leaving group stabilization during this step (Krooshof *et al.*, 1998). In reactions catalysed by serine hydrolases the carbonyl oxygen of the ester intermediate comes from the substrate which is significantly different from DhlA where the enzyme provides the oxygen resulting in a covalent ester intermediate. The resulting intermediate which is a covalent alkylenzyme to which the halide is still bound is hydrolysed by a molecule of water that is activated by His289 (Janssen, 2004).
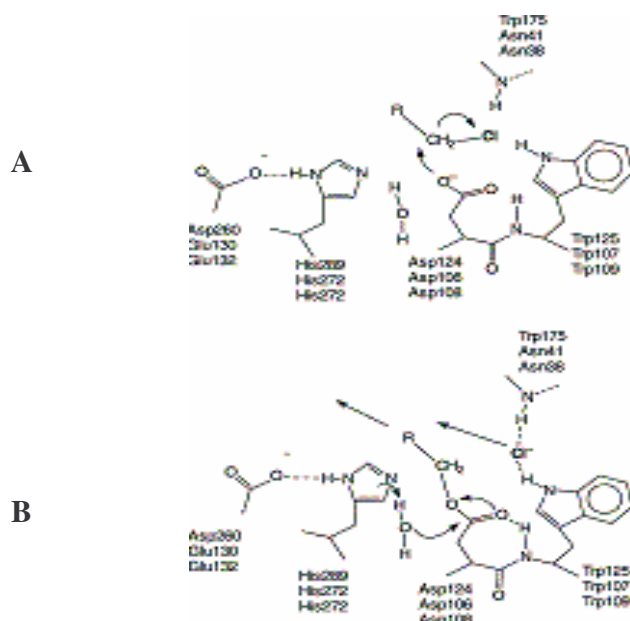
Fig. 1.13: General catalytic mechanism of haloalkane dehalogenases. (**A**) Formation of the covalent intermediate by S_N2 substitution. (**B**) Hydrolysis of the intermediate. Residue numbers refer to DhlA, DhaA and LinB, respectively (Janssen, 2004).

The stability of the positive charge that develops on His289 during the hydrolysis of the ester is maintained by the second aspartic acid of the catalytic triad. An oxyanion hole is formed by the main chain amides of Glu56 and Trp125, to which the non-reacting carboxylate oxygen of Asp124 remains hydrogen bonded allowing for the release of the halide from the active site (Fig. 1.13) (Verschueren *et al.*, 1993). Studies on the catalytic cycle were performed by soaking dehalogenase crystals with substrate at different pH values and solving the X-ray structures of the intermediates that accumulated. Stopped flow fluorescence measurements and rapid quench flow experiments were performed to study the kinetic mechanism of the *X. autotrophicus* GJ10 haloalkane dehalogenase (Janssen, 2004).

1.7   DIFFERENCES IN STUDIED HALOALKANE DEHALOGENASES

The structure of the two other haloalkane dehalogenases that have recently been solved: DhaA from a *R. erythropolis* NCIMB13064 (Newman *et al*., 1999) that breaks down 1-chlorobutane (Fig. 1.11a) and LinB from *S. paucimobilis* UT26 (Marek *et al*., 2000) that breaks down tetrachlorocyclohexadiene (Fig. 1.11c).   The substrate for LinB, tetrachlorocyclohexadiene has been proposed to undergo two consecutive dehydrodehalogenation steps to form the unstable intermediate hexachlorocyclohexane during the dehalogenation reaction (Marek *et al*., 2000). These enzymes are similar to DhlA in structure with the only differences being in geometry and size of the active site cavity and differences in the way in which the leaving group is stabilized (Janssen, 2004).

Five key residues make up the constitutional sequence of the catalytic machinery of the haloalkane dehalogenase and is therefore more properly called a catalytic pentad (Fig. 1.13). Basically it is composed of three residues that influence the nucleophilic attack in both chemical steps (equivalent to the catalytic triad in serine proteases) and a diad of two H-bond donating residues that are involved in leaving group stabilization in the first step. Within this pentad are three residues that are invariant in DhlA, DhaA and LinB both in identity and topological position in the structure (Fig. 1.12). The first is a nucleophilic aspartate located between β-strand 5 and α-helix 3 (Fig. 1.12) that is fully conserved. The second is a histidine residue that acts as a base in the catalytic cycle and is essential for the deprotonation of water during cleavage of the covalent intermediate which is conserved after β-strand 8. There is also a tryptophan residue flanking the

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nucleophilic aspartate that is involved in halide binding and is also invariable. However, the third member of the catalytic triad is variable as it can be an aspartate in DhlA located in the sequence distal from the cap domain after β-strand 7 or it can be a glutamate in LinB and DhaA, located before the cap after β-strand 6. There is one other halide stabilizing residue and this may also be variable. It could be a Trp located in the cap domain as in DhlA or an Asn located at a position proximal to the catalytic nucleophile after β-strand 3 and before α-helix 1 (Bohac *et al*., 2002 and Janssen, 2004).

When the structure of DhlA and DhaA are compared, there exists a 32% sequence identity and it appears that DhaA has a larger substrate binding cavity. It can also be seen that that the role of the acid residue is taken by Glu141 which occupies a position equivalent to Asn148 in DhlA (Bohac *et al*., 2002, Janssen, 2004 and Newman *et al*., 1999). When comparing the structure of the *Sphingomonas* haloalkane dehalogenase (LinB) to the other dehalogenases it can be seen that it is more similar to the DhaA of *Rhodococcus* dehalogenase (49% sequence identity) rather than the *Xanthobacter* enzyme (30% identity) (Kahn and Bruice, 2003). It is also evident that both the topological position of the catalytic triad residues (Asp108, His272, Glu132) and the position of the halide binding residues correspond to those of DhaA and this was confirmed by site directed mutagenesis (Bohac *et al*., 2002). Interestingly the active site cavity of LinB is larger compared to the other dehalogenases. This is in keeping with its remarkable role in tetrachlorocyclohexadiene dehalogenation as well as its activity with other cyclic substrates and linear primary and secondary haloalkanes. The presence of a

larger active site results in the exclusion of short chain chlorinated aliphatics such as DCA (Bohac *et al*., 2002).

1.8 BIOTECHNOLOGICAL APPLICATIONS OF BACTERIAL DEHALOGENASES

Microbial transformation of halogenated substances may be technologically used in two key processes. The first being synthesis where whole cells, crude cell preparations or purified enzymes may be used in the biosynthetic conversion of halogenated compounds to synthetic or novel intermediates [bioconversion]. The second process involves the use of halohydrocarbon-degrading bacteria or bacterial dehalogenases to detoxify and mineralize environmental pollutants [biodegradation] (Parales *et al*., 2002).

1.8.1 Microbial dehalogenases as industrial biocatalysts

Many of the currently used halogenated compounds are synthesized by chemical techniques. These methods that are generally expensive, result in the production of unwanted intermediates and sometimes result in the production of racemic mixtures. Biotransformation may provide an economic and versatile alternative. Currently several biotransformation processes are being employed that result in fairly enantio pure products (Swanson, 1999).

Short chain 2-halocarboxylic acids such as 2-monochloropropionic acid are widely used as intermediates in the production of pharmaceutical products and as key intermediates in the synthesis of phenoxypropionate herbicides. A racemic mixture of 2-monochloropropionate used for chemical synthesis will result in the production of racemic products with only one of the isomers being biologically active while the other would be expensive inactive chiral ballast or may even lead to toxic side effects. In this case the chiral specificity of 2-haloalkanoic acid dehalogenases is used to remove the L- or D-isomeric form from the chiral mixture due to the selective dehalogenation of one of the isomers (Motosugi *et al*., 1983). At a commercial scale *P. putida* AJ1/23 produces L-2-monochloropropionate for herbicide manufacture from racemic 2-monochloropropionate (Motosugi *et al*., 1983). There are several other examples where dehalogenases are used in the production of industrially important chemical intermediates (Swanson, 1999).

1.8.2   Environmental protection and waste management

Currently, most industrial processes have end-of-pipe or in-process treatment facilities for the handling of toxic material to avoid the production of hazardous material. Pesticides currently present a serious problem as their direct application can not only lead to contamination of soil but also ground water and aquifers through leachates. Improper disposal techniques in the past have resulted in a legacy of contaminated landfill sites, industrial sites and landfill leachates. In the development of efficient strategies for waste

management the use of microbial systems such as bacterial dehalogenases may become important tools used to deal with environmental pollutants (Fetzner and Lingens, 1994).

In order to use microbial systems in waste management strategies these microbes should detoxify, degrade or accumulate environmental pollutants. Strategies for waste management that biotechnology offers may include: (i) improvement of existing processes by application of adapted or engineered microbial strains (e.g. the municipal treatment of wastewaters); (ii) use of adapted or genetically engineered microorganisms to treat contaminated soil, groundwater or aquifers; (iii) construction of bioreactors containing biofilms of suitable organisms or use of immobilized biocatalysts in the detoxification of environmental chemicals; (iv) development of biosensors to detect trace amounts of toxic organics or heavy metals and (v) recovery of products from wastes (e.g. recovery of metals by using metal accumulating bacteria. Successfully applied biotechnological approaches have thus far involved bioaugmentation to cope with contaminated landfill sites, industrial sites and groundwater and the development of bioreactors to deal with specific contaminants (Fetzner and Lingens, 1994).

A pollutant may persist in the environment for one of several reasons and these may include: (i) the absence of microorganisms with the capability of degrading the compound; (ii) the presence of unfavourable environmental conditions for degradation [environmental factors which may influence biodegradation include permeability of the subsurface for air and water, temperature, pH, salinity and water content (water activity), oxygen tension (redox potential) availability of nutrients, presence of inhibitory

chemicals and the presence of competing organisms or of predators (protozoa) grazing on the degradative microorganisms]; (iii) unfavourable substrate concentration (the pollutant may be present in too high a concentration, leading to toxicity or in too low a concentration failing to induce the degradative enzymes or being below the threshold concentration and (iv) lack of bioavailability of the pollutant because of incorporation into humic substances or strong adsorption to soil particles. For any bioremediation strategy to be employed successfully the environmental conditions must thus be optimized so that the indigenous or inoculated microorganisms can degrade the pollutant at the maximum possible rate (Fetzner and Lingens, 1994).

1.9   SCOPE OF THE PRESENT STUDY

In recent times the increased reliance of industrial processes for the manufacture of synthetic products has resulted in the development of several synthetic chemical intermediates that are usually toxic to both man and the environment. Poor handling, disposal and accidental spillages have caused severe contamination of both terrestrial and aquatic ecosystems. This is of serious concern as many of these xenobiotic compounds are recalcitrant within the environments to which they are introduced. One of the widely synthesized compounds is DCA which is used in a variety of chemical processes. The compound is not only a suspected carcinogen in humans but it is also hazardous to the environment.

In recent times DCA has also become a priority pollutant in South Africa, therefore this study set out to isolate and characterize indigenous microorganisms that are capable of DCA degradation. The study has also go on to characterize the genes involved in the degradation process using specific gene probes that have been amplified from a known DCA degrading microorganism. Enzymatic studies on the breakdown of the different intermediates in the mineralization pathway have been used to determine the catabolic route and comparisons have been made to previously characterized bacteria. Phylogenetic studies using the pulsed field gel electrophoresis (PFGE) technique and the random amplified polymorphic DNA (RAPD) technique have been used to genotypically compare the new isolates and determine whether they are distinct from each other.

1.9.1 Hypothesis to be tested

It is hypothesized that the isolation and characterization of bacterial isolates capable of DCA degradation may assist in an understanding of how degradative genes are globally disseminated. It is further hypothesized that the characterization of the genes involved in the degradation processes may help in understanding how these microorganisms are capable of completely degrading DCA.

1.9.2 Objectives

The following objectives were established to test the above hypothesis:

(a)   to determine whether bacteria capable of DCA degradation are present in South African waste water treatment facilities;

(b)   to determine which genes are involved in the degradation process;

(c)   to identify the catabolic route involved the mineralization of DCA; and

(d)   to determine whether molecular typing techniques can be used to assess the level relatedness between the different isolates.

1.9.3 Experimental design

In order to achieve the stated objectives, the following aims were pursued in the chapters indicated:

Chapter Two:

a)   to obtain wastewater samples from various sources;

b)   to isolate bacteria by selective enrichment with DCA;

c)   to determine if the bacterial isolates were capable of halide release;

d)   to construct bacterial growth curves of bacteria capable of utilizing DCA as a carbon source;

e)   to identify dehalogenase genes by PCR; and

f)   to identify bacterial isolates by 16S rDNA sequencing.

Chapter Three:

a)   to amplify the dhlA gene;

b)   to clone and sequence the dhlA gene;

c)   to perform Southern transfer of restricted genomic DNA;

d)   to hybridize a dhlA labeled probe to membranes containing DNA;

e)   to PCR amplify the aldA and aldB genes;

f)   to hybridize an aldB labeled probe to membranes containing DNA;

g)   to PCR amplify the transposase gene;

h)   to hybidise a labeled transposase gene probe to membranes containing DNA;

i)   to PCR amplify the dhlB gene;

j)   to hybidise a labeled dhlB gene probe to membranes containing DNA; and

k)   to compare gene organization using different primer combinations.

Chapter Four

a)   to prepare crude cell lysates from bacterial isolates;

b)   to determine total protein in crude cell lysates;

c)   to determine haloalkane dehalogenase activity from crude cell lysates;

d)   to determine haloalcohol dehydrogenase activity from crude cell lysates;

e)   to determine aldehyde dehydrogenase activity from crude cell lysates;

f)   to determine haloacetate dehalogenase activity from crude cell lysates; and

g)   to perform PAGE analysis of total protein.

Chapter Five

a)   to perform PFGE on restricted genomic DNA;

b)   to perform RAPD analysis using specific primers; and

c)   to compare results obtained by PFGE and RAPD analysis.

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CHAPTER TWO

ISOLATION AND CHARACTERISATION OF 1,2-DICHLOROETHANE (DCA) DEGRADING BACTERIAL ISOLATES FROM SOUTH AFRICAN WASTE WATER WORKS

2.1   INTRODUCTION

Halogenated organic compounds constitute one of the largest groups of environmental pollutants as a result of their widespread use as herbicides, insecticides, fungicides, solvents, hydraulic and heat transfer fluids, plasticizers and intermediates for chemical synthesis.   Because of their potential toxicity, bioconcentration and persistence; the ubiquitous distribution of halogenated compounds in the biosphere has caused public concern over the possible effects on the quality of life (Fetzner and Lingens, 1994).

The biosphere contains a multitude of halogenated organic compounds, more than 2400 of which have been identified as occurring naturally; however, those constituting the bulk quantities are synthesized industrially (Hill *et al*., 1999). Many halo-organic compounds have been categorized as priority pollutants even though a wide range of bacterial species that can degrade such substances, and in many cases utilize them as sole sources of carbon and energy, have been isolated in laboratory culture (Fetzner and Lingens, 1994). Notwithstanding the recalcitrance of halo-organic compounds in the biosphere, microbial catabolism is clearly a major latent route by which these compounds may be detoxified

and recycled. Therefore, there is the need to understand much more about the process of microbial adaptation involved in the degradation process in order to harness this potential (Hill *et al*., 1999).

The suspected carcinogenic solvent 1,2-dichloroethane (DCA) is one of the most abundant chlorinated groundwater pollutants which has been detected at up to 65µg/L drinking water. DCA is a xenobiotic compound used in the degreasing of metals as well as in the production of various plastics. This compound is not easily degraded in the environment and its solubility in water (8.69 X 10^3mg/L at 20°C) allows it to contaminate groundwater supplies, thus affecting all forms of life (National Toxicology Program, 2005). DCA has been found to leach rapidly into groundwater and have detected up to 2500 mg/L (Hunkeler *et al*., 2005) where degradation rates are low and the half life of DCA may increase from 1 year to 23 years (Barbash and Reinhard, 1989). The good solubility (8.69 X 10^3mg/L at 20°C) and low Hendry's Law Co-efficient (0.00118 atm-m^3/mole) enables DCA to permeate both into the water systems and the atmosphere where abiotic factors have also been shown to influence the degradation of DCA (Barbash and Reinhard, 1989).

Bioremediation of chlorinated aliphatic compounds may be described as the process by which microorganisms metabolise these compounds either through oxidative or reductive processes. The aerobic oxidative conversion of these compounds into non-toxic by-products such as carbon dioxide, water and chloride ions or organic acids, methane and chloride ions in the case of anaerobic reductive conversion would only occur under

favorable conditions. A typical bioremediation process may be directed towards : (a) the complete oxidation of organic contaminants (termed mineralization); (b) the biological transformation into less toxic metabolites (biotransformation) or the reduction of certain electrophilic groups by transferring electrons from electron donors (sugars or fatty acids) to the contaminant thereby resulting in it becoming less toxic (Head, 1998).

Bioremediation may be achieved by the enhancement of natural processes such as the addition of microorganisms to the soil referred to as bio-augmentation or alternatively the addition of amendments such as oxygen, moisture or nutrients and providing suitable conditions for the growth of microorganisms naturally present in the soil referred to as bio-stimulation (Head, 1998). In addition, efficient degradation includes factors such as the bioavailability of the compound and the ability of the substrate to diffuse or be transported into the cell (Pieper and Reineke, 2000).

Microorganisms are very versatile, diverse and possess the ability to easily adapt to new environments which makes them the most suitable candidates to redirect xenobiotic compounds into biogeochemical cycles. In recent years there has been a flurry into research to isolate new microorganisms that possess unusual catabolic activities that can be used to degrade anthropogenic compounds however some compounds have been shown to be unusually recalcitrant i.e microorganisms either do not metabolize these compounds or do not posses the necessary enzymes to transform these compounds (Eyers *et al*., 2004).

It has also become evident in recent times that the degradation pathways of many xenobiotics are to an extent poorly characterized or not understood at all. One of the possible reasons for this could be the constant isolation of the same species whenever attempts are made to cultivate pollutant degrading microorganisms (Eyers *et al*., 2004). Techniques used for the cultivation of microorganisms are constantly improving but a larger proportion of earths' microbes remains uncultured due to the inability to isolate and cultivate them in/on appropriate media (Leadbetter, 2003).

Xanthobacter autotrophicus GJ10 was the first bacterium isolated that is capable of utilizing DCA as a sole carbon and energy source. This bacterium was isolated from a mixture of activated sludge from a waste water treatment plant and soil samples obtained from various chemically polluted sites (Janssen *et al*., 1984). The isolation procedure involved the repeated sub-culturing of a minimal salts medium inoculated with the sludge and soil samples, and supplemented with vitamins and 5mM DCA. The utilization of DCA as a carbon source was indicated by an increase in turbidity and chloride concentration over a 5 week period following repeated sub-culturing into fresh medium supplemented with vitamins and 5mM DCA (Janssen *et al*., 1984).

Isolates of *Ancylobacter aquaticus* capable of DCA utilization were later obtained from brackish water sediment taken from the Eems channel near Delfzijl, The Netherlands, following enrichment with DCA or chloroethylvinyl ether as growth substrates (van den Wijngaard *et al*., 1992). Song *et al*., 2004 later isolated *X. flavus* UE15 from waste water and rice paddy soil samples from South Korea when DCA was used as the sole carbon

and energy source. All of these isolates metabolized DCA through a similar catabolic route with the final product, glycolic acid entering the cell's central metabolic pathway to be used as a carbon and energy source (Janssen *et al.*, 1984; Song *et al.*, 2004; van den Wijngaard *et al.*, 1992).

The hydrolytic haloalkane dehalogenase was first identified in *X. autotrophicus* GJ10 (Janssen *et al.*, 1989) and later identical DhlA encoding genes were identified in isolates of *A. aquaticus* (van den Wijngaard *et al.*, 1992) as well in *X. flavus* UE15 (Song *et al.*, 2004). This enzyme is the first enzyme in the DCA catabolic pathway and is at present the only hydrolytic haloalkane dehalogenase that is present in DCA degrading bacteria with no variants having been described (Janssen *et al.*, 2005).

Sphingomonas paucimobilis UT26 formally *Pseudomonas paucimobilis* UT26, isolated by Imai *et al.* (1989) is capable of degrading the toxic organic halogenated insecticide, γ-hexachlorocyclohexane (lindane). This isolate was obtained from soil contaminated with this persistent pesticide through repeated sub-culturing using lindane as a carbon source. The gene coding for the hydrolytic dehalogenase (*linB*) was later identified (Nagata *et al.*, 1993). Verhagen *et al.* (1995) found that 1,3-dichloropropene, a widely used nematocide against root knot nematodes was more rapidly degraded after regular applications while degradation occurred at a slower rate in similar soil types not previously exposed to 1,3-dichloropropene. The ability of soils to adapt in order to accelerate degradation of pesticides was attributed to the selection of microbial populations with enhanced degradative capacities. The use of gene probes and enrichment resulted in the isolation

of the 1,3-dichloropropene degrader, *Pseudomonas pavonacae* 170 formally *P. cichorii* 170.

The hydrolytic dehalogenase gene (*dhaA*) responsible for the degradation of 1,3-dichloropropene was later characterized by Poelarends *et al*. (1998). It was also found that a homologous gene was also present in a totally unrelated bacterium, *Rhodococcus erythropolis* NCIMB13064 formally *R. rhodochrous* NCIMB13064 (Kulakova *et al*., 1997). Database searches of sequenced genomes resulted in the identification of a hydrolytic dehalogenase type gene (*dhmA*) from *Mycobacterium tuberculosis* H37Rv (Jesenska *et al*., 2000). Expression studies showed that hydrolytic dehalogenase coded for by this gene was indeed capable of catalyzing the cleavage of carbon-halogen bonds (Jesenska *et al*., 2002).

Once a suitable candidate capable of degrading or transforming a particular xenobiotic compound has been isolated it is important to identify the isolate and assign it to a genus or sub-species. It is generally difficult to identify slow growing and fastidious microorganisms using traditional phenotypic methods which may require a degree of subjective judgment (Drancourt *et al*., 2000). Currently, the use of molecular techniques for the identification has proved to be very useful because there is no prior requirement to culture the microorganisms under several different conditions (Marchesi *et al*., 1998).

The use of the rRNA gene sequence as taxonomic tool has completely redefined phylogenetic relationships that were previously too dependant on cellular metabolism (Fox *et al*., 1980). It has also been found that in addition to the highly conserved regions within the 16S rRNA gene sequence there is sufficient variation that is useful in the differentiation of genera and species (Jensen *et al*., 1993). Phylogenetic relationships of bacteria and all forms of life could be shown by comparing a stable part of the genetic code. The genetic area that could be compared in bacteria included the genes that code for the 5S, 16S (also called the small sub-unit) and the 23S rRNA and the spaces between these genes. The 16S rRNA gene which is also designated 16S rDNA is now commonly used interchangeably for taxonomic purposes although current ASM policy is that term 16S rRNA gene be used (Clarridge, 2004).

This chapter focuses on the isolation of DCA degrading bacteria from different waste water treatment works in the KwaZulu-Natal region using specific enrichment techniques with DCA being the sole carbon and energy source. The gene coding for the hydrolytic dehalogenase involved in the cleavage of the carbon-halogen bond at the very beginning of the DCA catabolic route was amplified by primers designed specifically for the amplification of hydrolytic dehalogenases. The growth patterns of the isolates were compared when grown in a minimal medium containing DCA as a sole source of carbon and energy. A preliminary identification of all new isolates was carried out by amplifying, cloning and sequencing the 16S rDNA genes (Marchesi *et al*., 1998).

2.2   MATERIALS AND METHODS

2.2.1   Bacterial strains used in this study

Table 2.1:   Hydrolytic dehalogenase producing bacteria used in this study

Bacterial strain	Source
Pseudomonas pavonaceae 170 formally *P. cichorii* 170	D. B. Janssen*
Rhodococcus erythropolis NCIMB13064 formally *R. rhodochrous* NCIMB13064	D. B. Janssen*
A. aquaticus AD25	D. B. Janssen*
A. aquaticus AD27	D. B. Janssen*
X. autotrophicus GJ10	D. B. Janssen*

* D. B. Janssen: Biochemical Laboratory, Biomolecular Sciences and Biotechnology Institute, University of Groningen, Groningen, The Netherlands.

2.2.2   Sample collection

The Northern Waste Water Works (KwaZulu-Natal) was identified as one of the waste water facilities that handled large quantities of industrial waste water. Samples were collected in sterile Schott bottles from various treatment points which included the settling tank, activated sludge treatment, secondary clarifier, and anaerobic sludge digester. Samples were also collected from SAPPI aerated paper treatment works and SAPPI bleach plant (South African Pulp and Paper Industry, KZN). All samples were transported on ice and stored at 4˚C until further use.

2.2.3   Media and growth conditions

Minimal salts medium was prepared per litre as follows: 1.36g KH_2PO_4; 5.37g $Na_2HPO_4.12H_2O$; 0.5g $(NH_4)_2SO_4$; 0.2g $MgSO_4.7H_2O$ (sterilized by autoclaving at 121˚C for 15min) and 5ml of a trace metal solution (added after filter sterilization). The trace metal solution contained per litre: 530mg $CaCl_2$; 200mg $FeSO_4.7H_2O$; 10mg $ZnSO_4.7H_2O$; 10mg H_3BO_3; 10mg $CoCl_2.6H_2O$; 4mg $MnSO_4.5H_2O$; 3mg $Na_2MoO_4.2H_2O$ and 2mg $NiCl_2.6H_2O$. The trace metal solution was filter sterilized through a 0.22µm syringe filter (Millipore) and stored at 4˚C. Prior to inoculation this medium was supplemented with 1ml vitamin stock solution which contained per litre: 12mg biotin; 1g choline chloride; 1g calcium (D)-pantothenate; 2g i-inositol; 1g nicotinic acid; 1g pyridoxine chloride; 1g thiamine chloride; 0.2g p-aminobenzoic acid and 0.01g cyanocobalamin (Janssen *et al.*, 1984). This medium was supplemented with syringe filter sterilized (Millipore) 5mM DCA (final concentration) (Aldrich) which served as the carbon and energy source. The pH of the medium was adjusted to 7 prior to inoculation. The volume of the complete medium was adjusted to 1L with deionised water. Fifty milliliters of the minimal salts medium was then aliquoted into 250 ml Schott bottles with air tight viton rubber seals. Each bottle was inoculated with 1% inoculum from each of the sources. Bottles were incubated at 30˚C with shaking. Fresh medium was inoculated with 1% inoculum from these bottles every five days over a 25-day period.

2.2.4   Isolation of pure cultures

Following a 25-day enrichment, 100µl of culture was spread over minimal salts agar medium. The agar medium (1l) was prepared by first sterilizing 1.36g KH_2PO_4; 5.37g

Na$_2$HPO$_4$.12H$_2$O; 0.5g (NH$_4$)$_2$SO$_4$; 0.2g MgSO$_4$.7H$_2$O together with 12g bacteriological agar. This was then allowed to cool to approximately 45˚C and 5ml of the trace metal and 1ml of the vitamin solutions (Section 2.2.3) were then added, and poured into 90mm Petri plates and allowed to solidify. After inoculation the plates were inverted and 20µl of DCA was added to filter paper discs placed on the inside surface on the lid (Janssen *et al*., 1989). This allowed for the creation of a DCA atmosphere. Plates were sealed with cellophane tape and incubated at 30˚C until colonies appeared. Single colonies were then transferred to Luria Bertani (LB) agar (10g tryptone, 5g yeast extract and 12g bacteriological agar per litre) plates without the addition of NaCl.

2.2.5   Halide release assays

In order to determine whether the individual isolates did possess the ability to release free halide from DCA a simple qualitative colorimetric assay was performed (Janssen *et al*., 1989). To a standard clear round bottom microtitre plate 100µl of 50mM Tris-sulphate (pH7.5) containing 5mM 1,2-dibromoethane was added. Individual colonies were then picked up using a needle and emulsified into the substrate solution. The plate was then incubated for 1h at 30˚C. Following incubation, 100µl of 0.25M ferric ammonium sulfate [Fe(NH$_4$)(SO$_4$)$_2$.12H$_2$O] solution in 6M nitric acid was added followed by one drop of a saturated solution of mercuric thiocyanate in ethanol. The development of a red colour is indicative of the presence of free halide. For the halide release assays; *X. autotrophicus* GJ10 was used as a positive control while *E. coli* DH5α was used as a negative control.

2.2.6   Comparison of bacterial growth

In order to determine whether the bacterial isolates do indeed utilize DCA as a sole carbon and energy source, individual isolates were re-inoculated into the minimal salts medium containing 5mM DCA and growth was monitored spectrophotometrically. This was achieved by first growing the individual isolates in 100ml LB broth overnight at 30°C with shaking. Cells were then harvested by centrifugation at 10 000 x *g* for 10min. Cells were then washed once in an equal volume of saline (0.87g NaCl/l, pH7) and finally re-suspended in 20ml saline. The optical density of cells were then adjusted to 0.05 at 600nm. Schott bottles (250ml) containing 50ml minimal salts medium together with 5mM DCA with a viton sealed hole in the cap through which sample can be withdrawn were then inoculated with 500µl cell suspension. The optical density at 600nm was monitored at various time intervals until stationary phase was achieved.

2.2.7   Dehalogenase gene identification

2.2.7.1   Primer design

PCR primers were designed based on the sequences of known hydrolytic dehalogenase genes. Sequences were downloaded from the NCBI Database and aligned using the DNAMAN DNA analysis software (Lynon Biosoft). The DNA sequences of the *dhlA* gene (accession number: M26950) from *X. autotrophicus* GJ10, the *linB* gene (accession number: D14594) of *S. paucimobilis* UT26, the *dhaA* gene (accession number: AJ250371) from *P. pavonaceae* 170 and *R. erythropolis* NCIMB13064; and the *dhmA* gene from *Mycobacterium tuberculosis* H37Rv (accession number: Z77724) were aligned. It was found that the *dhaA* and *linB* genes showed 56% identity therefore these

were aligned to form the LIN primer set. The remaining two genes *dhlA* and *dhmA* were found to have 50% identity therefore these were aligned to form the DHM primer set. All alignments were performed using the DNA analysis software DNAMAN (Lynon Biosoft). The primers were designed based on regions showing a significant level of identity and suitably far apart to generate a PCR product (Table 2.2). The *dhlA* gene of *X. autotrophicus* GJ10 and *A. aquaticus* AD27 are identical (van den Wijngaard *et al*., 1992) and will be used as controls for amplification of the *dhlA* gene. The *dhaA* genes of *P. pavonaceae* 170 and *R. erythropolis* NCIMB13064 are identical (Poelarends *et al*., 1998) and would be used as controls for the *dhaA* gene. The expected size of the PCR product in the control bacteria using either primer set is 450bp. Primers were synthesized by Inqaba Biotech (South Africa).

Table 2.2:   Primers designed for the amplification of hydrolytic dehalogenases

Primer	Based on alignment of genes-	Sequence of primers*
DHM	*dhlA* and *dhmA*	F: GGCGAGCCCACCTGGAGYTAC
		R: GWMKYGTCRGGGAARGGCGC
LIN	*linB* and *dhaA*	F: CTGTGGCGCAAYATCATSCCG
		R: GAGGAAGGGCTCGCGATAGKSGKC

*IUPAC ambiguity code used: B = C, G, or T; D = A, G, or T; K = G or T; M = A or C; N = A, C, G, or T; R = A or G; S = C or G; W = A or T; Y = C or T.

2.2.7.2   DNA amplification

Total DNA was isolated using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturers' instructions and quantified using the GeneQuant, RNA/DNA Calculator (Pharmacia). Stock solutions of primers were prepared by the addition of an appropriate

amount of sterile de-ionised water to create a 1M stock while working stocks were at 100mM. Both primer stocks were stored at -20˚C. The PCR mixtures (50µL) contained 10 ng DNA, 100 pmol of each of the appropriate primers, 100 µM of each of the deoxynucleoside triphosphates (dNTPs), 1 x Super-therm *Taq* DNA polymerase buffer and 0.5 U Super-therm *Taq* DNA polymerase (Southern Cross Biotech) finally brought to volume with sterile de-ionised water. PCR was performed using the PE Applied Biosystems GeneAmp PCR System 9700 (Perkin Elmer) programmed to perform an initial denaturation at 94˚C for 5min and 30 cycles consisting of 94˚C for 2min, 60˚C for 1min and 72˚C for 2min followed by a final extension step of 72˚C for 5min. The PCR conditions were the same for both primer sets. DNA from *X. autotrophicus* GJ10 was used as a positive control for the DHM primer set (*dhlA*) and DNA from *P. pavonaceae* 170 was used as positive control for the LIN primer set (*dhaA*). PCR products were identified by agarose gel electrophoresis on a 2% TAE agarose gel (Sambrook *et al*., 1989). Gels were documented and analysed using the Chemi Genius Bio Imaging System (Syngene).

2.2.8 Bacterial identification

2.2.8.1 16S rDNA sequencing

Total DNA used in all PCR reactions were isolated using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturers' instructions. The 16S rDNA regions were amplified by PCR as described by Marchesi *et al*. (1998). Following amplification, PCR products were visualized on a 2% agarose gel. PCR products were cleaned using the QIAquick PCR Cleanup Kit (Qiagen). Cleaned products were ligated to the pGEM-T

Easy Vector System I (Promega) and electro-transformed into electro-competent *E. coli* DH5α cells. Transformants were selected on LB agar (10g tryptone, 5g yeast extract, 5g NaCl and 12g bacteriological agar per litre) plates containing 100µg/ml ampicillin. Selection of vectors containing inserts was carried out by spreading 20µl of 1M IPTG and 50µl of a 20mg/ml stock of X-gal onto plates prior to inoculation. Positive transformant colonies (cream in color) were grown overnight in 3ml LB broth (10g tryptone, 5g yeast extract, 5g NaCl) containing 100µg/ml ampicillin. Plasmid DNA was isolated using the High Pure Plasmid Isolation Kit (Roche) and sequenced by Inqaba Biotech from both directions using the T7 and SP6 primers. DNA sequences were edited and aligned using DNAMAN DNA analysis software (Lynon Biosoft). The BLAST program (Altschul *et al*., 1997) was used to screen DNA databases for sequences that share similarity with new sequence information. DNA sequences were aligned using Genedoc version 2.4 (Nicholas and Nicholas, 1997).

2.2.9   Biochemical tests and Gram staining

The following biochemical tests were carried out according to standard procedures: nitrate reduction test, catalase test, oxidase test and carbohydrate fermentation test (Cowan and Steel, 1985). Gram staining was performed in order to determine bacterial morphology.

2.3 RESULTS

2.3.1 Isolation of halide releasing bacteria

The turbid appearance of the minimal following repeated sub-culturing every five days over a 25 day period indicated the presence bacteria capable of DCA utilization. A total of 187 bacterial isolates were screened using the halide release assay. These isolates appeared as single colonies on the minimal salts agar plates and were subsequently transferred to LB without NaCl before being screened. Distinct colonies were chosen from the different sources based on colour, size of colony and colony morphology. Five isolates from different point sources produced a positive result for the halide release assay. Three isolates (DH2, DH5 and DH12) were isolated from different points at the Northern Waste Water Works while two other isolates (UV5 and UV6) were isolated from different points at SAPPI waste water treatment facility.

2.3.2 Comparison of bacterial growth

Fig. 2.1 shows the growth curves of the different isolates in a minimal salts medium containing 5mM DCA as a carbon source. The increase in the absorbance over time indicates that these isolates have the ability to utilize DCA as carbon and energy source because controls without DCA showed no increase in biomass. *A. aquaticus* AD27, a known degrader of DCA which was used as a control did not perform as well as isolates DH2, DH5 and DH12. All of the isolates show a relatively long lag period of approximately 24 hours and stationary phase is generally reached after 36 hours (except

for isolate UV5 which has a longer exponential phase and stationary phase is only reached after 48 hours).
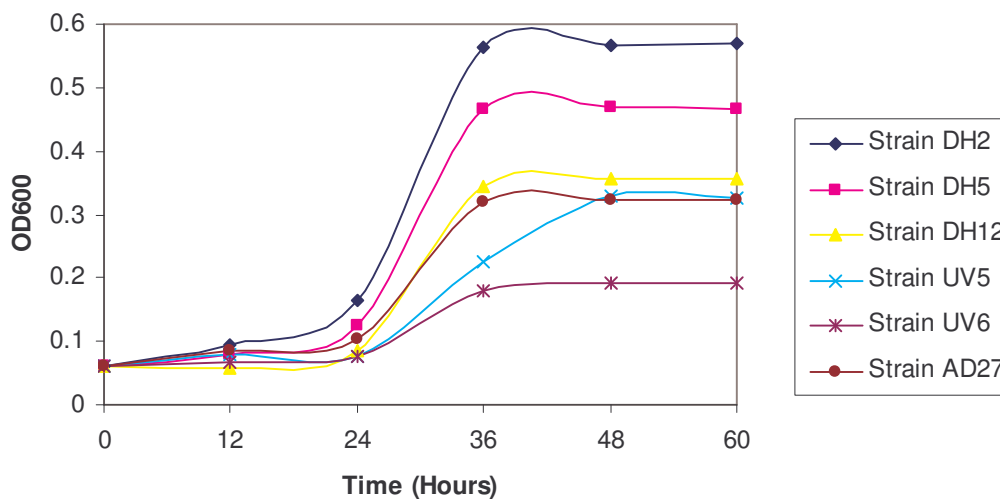
Fig. 2.1:   Bacterial growth curves of the different isolates grown in minimal salts medium containing 5mM DCA. *A. aquaticus* AD27 was used as a control organism.

2.3.3   Dehalogenase gene identification

The DHM and LIN PCR primer sets (Table: 2.2) were used in order to determine to which group of hydrolytic dehalogenase producing bacteria the South African isolates belonged. The 450bp region of the *dhaA* gene was amplified in *P. pavonacae* 170 (lane 9) and *R. erythropolis* NCIMB13064 (lane 10) whereas no products were observed for the South African isolates as well as *X. autotrophicus* GJ10 and *A. aquaticus* strains AD25 and AD27 (lanes 1 to 8) using the LIN primer set (Fig. 2.2). This may indicate that the South African isolates may not possess a dehalogenase gene that is related to the *dhaA*. The inability of the LIN primer set to amplify any part of the dhlA gene of *X. autotrophicus* GJ10 and *A. aquaticus* strains AD25 and AD27 (lanes 1 to 8) indicates that this primer set is specific for the *dhaA* gene.

Fig. 2.3 shows amplification of the 450bp region of the *dhlA* gene of *X. autotrophicus* GJ10 and *A. aquaticus* AD27 (which were used as controls) as well the appearance of a PCR product of similar size for the South African isolates using the DHM primer set (lanes 1 to 8). There were no products observed for *P. pavonacae* 170 (lane 9) and *R. erythropolis* NCIMB13064 (lane 10). The amplification of a region of similar size within the genomes of the South African isolates of *A. aquaticus* may indicate that these isolates posses the *dhlA* gene. The DHM primer is specific for the 450bp region within the *dhlA* gene as no products were observed for *P. pavonacae* 170 (lane 9) and *R. erythropolis* NCIMB13064 (lane 10).
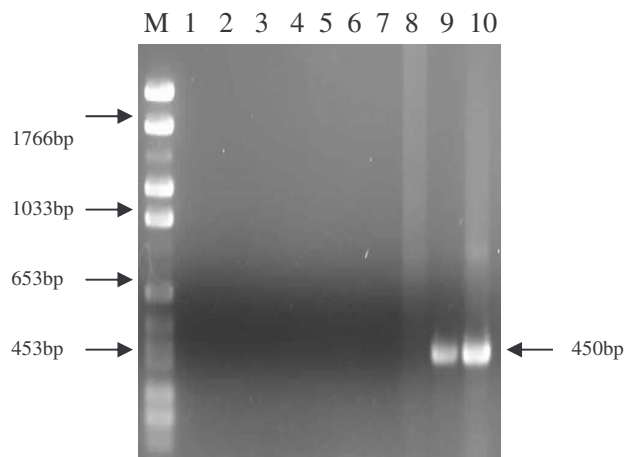
Fig. 2.2: PCR amplification using the LIN primer set. M: Marker VI; Lane1: DH2; lane 2: DH5; lane 3: DH12; lane 4: UV5; lane 5: UV6; lane 6: *A. aquaticus* AD25, lane 7: A. *aquaticus* AD27; lane 8: *X. autotrophicus* GJ10; lane 9: *R. erythropolis* NCIMB13064 and lane 10: *P. pavonacae* 170.
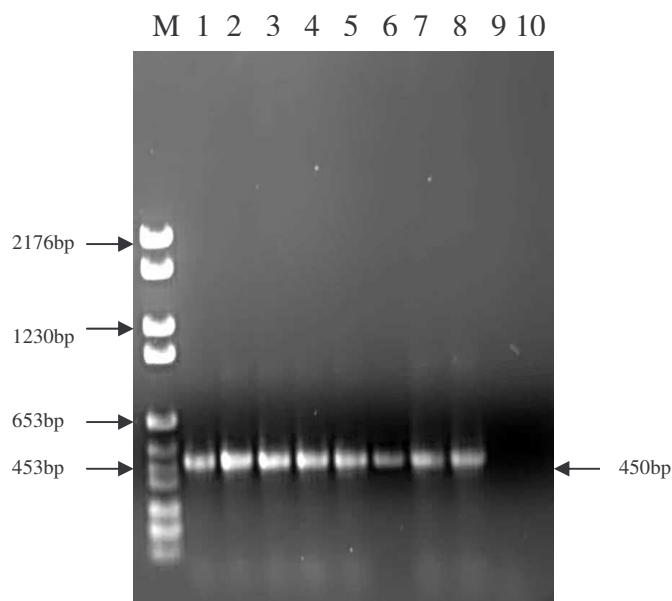
Fig. 2.3: PCR amplification using the DHM primer set. M: Marker VI; Lane1: DH2; lane 2: DH5; lane 3: DH12; lane 4: UV5; lane 5: UV6; lane 6: *A. aquaticus* AD25, lane 7: A. *aquaticus* AD27; lane 8: *X. autotrophicus* GJ10; lane 9: *R. erythropolis* NCIMB13064 and lane 10: *P. pavonacae* 170.

2.3.4. Bacterial identification

2.3.4.1   16S rDNA sequencing

Fig. 2.4 shows the successful amplification of the partial 16S rDNA region of the South African isolates.   The amplicons produced are of the correct size as indicated by Marchesi *et al*. (1998).
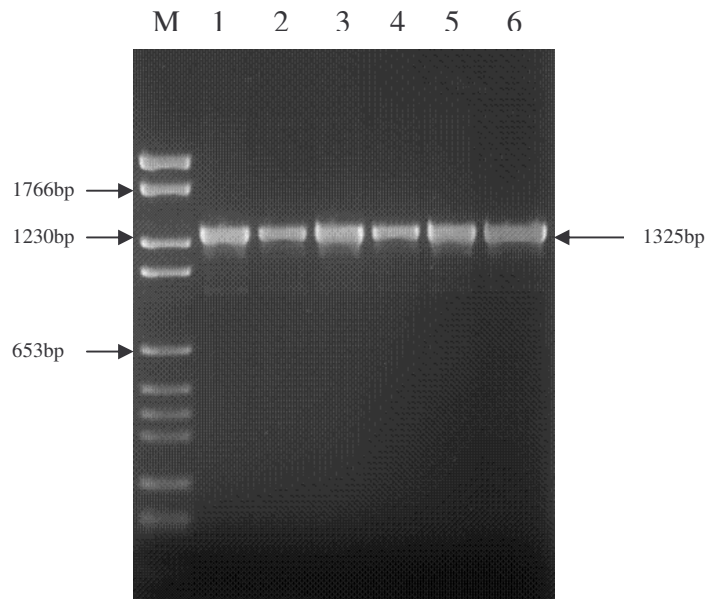
Fig. 2.4: PCR amplification using the 16S rDNA primer set. M: Marker VI; Lane1: DH2; lane 2: DH5; lane 3: DH12; lane 4: UV5; lane 5: UV6; lane 6: *A. aquaticus* AD27.

Plasmid preparations were successfully sequenced by Inqaba Biotech. Following BLAST analysis of edited sequence information all of the isolates were shown to be different strains of *Ancylobacter aquaticus* with at least 99%sequence identity. Aligned sequence information appears in Appendix A.

2.3.5   Biochemical tests and Gram staining

Results of the biochemical tests indicated that all isolates were oxidase and catalase positive. The isolates did not show the ability to fix nitrogen or reduce nitrate. The isolates were able to utilize glucose ad galactose as carbon sources but not lactose and citrate. Gram staining indicated that all isolates were Gram negative and had a horse shoe shape typical of the genus *Ancylobacter*.

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2.4 DISCUSSION

Janssen *et al*. (1984) described the isolation of the first bacterium, *X. autotrophicus* GJ10, capable of aerobic utilization of DCA as well as several other short chain aliphatic compounds as sole sources of carbon and energy. The gene coding for the hydrolytic dehalogenase (*dhlA*) capable of cleaving the carbon-chlorine bond was later cloned and sequenced (Janssen *et al*., 1989). It was later discovered that three isolates of *Ancylobacter aquaticus* (AD20, AD25 and AD27) also possessed the *dhlA* gene and were also capable of catalysing the breakdown of short chain aliphatic compounds (van der Wijngaard *et al*., 1992).

In this study waste water treatment plants handling chemical effluent were used as point sources for the isolation of bacteria capable of utilizing DCA as a sole carbon and energy source. The successful isolation of five isolates (DH2, DH5, DH12, UV5 and UV6) that were positive for a halide release assay indicated that DCA degrading bacteria are indeed present within the different waste treatment facilities.

Effluent treatment plants generally posses a great diversity of microorganisms co-existing as a dynamic community with each microbe playing a sometimes small but pivotal role in the degradation process (Rani *et al*., 2008). The bleaching of pulp by the pulp and paper industry involves the oxidation of wood pulp with elemental chlorine or chlorine oxide making this industry a major source of chlorinated organics which has now been substituted with H_2O_2 in Europe (Rahmawati *et al*., 2005). The waste water generated

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generally contains sugars, polysaccharides, organic acids, resin acids, lignin transformation products and a variety of chlorinated derivatives which is generally treated in aerated stabilization basin or lagoons making it the ideal source of chlorinated hydrocarbon degrading bacteria (Fulthorpe *et al*., 1992). Isolates DH2, DH5 and DH12 were isolated from different points of Northern Waste Water Works (KZN) which handles the treatment of industrial effluent whereas isolates UV5 and UV6 were isolated from the waste water treatment facility at SAPPI indicating that each of these facilities is a potential reservoir of microorganisms that have the capacity to be used in bioremediation strategies.

The isolation procedure used in this study entailed first enriching for DCA utilizing bacteria in a minimal salts broth medium supplemented with vitamins and DCA, followed by plating on a similar agar medium but replacing DCA incorporation into the medium with a DCA atmosphere. This two stage procedure ensured the selection of only bacteria capable of utilizing DCA as a carbon and energy source. The halide release assay initially developed by Bergmann and Sanik (1957), and later modified by Janssen *et al*. (1989) provides a simple means of detecting liberated chloride ions in solution. Although this assay was used to qualitatively detect free halide, it can be modified to quantify the liberated chloride ions.

In order to confirm that these isolates were indeed capable of utilizing DCA as sole carbon and energy source, pure cultures were re-inoculated into the mineral salts medium with DCA as the sole carbon source. For this experiment a known DCA degrader, *A*.

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aquaticus AD27 (van der Wijngaard *et al.*, 1992) was used as a control strain. From the results obtained (Fig. 2.1) it can be seen that three of the South African isolates (DH2, DH5 and DH12) were capable of better growth compared to *A. aquaticus* AD27 in the minimal medium containing 5mM DCA when standardized cultures were used.

These isolates may grow better in the presence of DCA compared to *A. aquaticus* AD27 because the Northern Waste Water Works (KZN) routinely handles large quantities of toxic industrial effluent which may give these isolates an adaptive advantage (Jain *et al.*, 2005) compared to *A. aquaticus* AD27 which was isolated from brackish water sediment following enrichment with DCA as a growth substrate (van den Wijngaard *et al.*, 1992).

PCR primers used for the amplification of regions within hydrolytic dehalogenase genes were designed based on currently available sequences of the known hydrolytic dehalogenase genes. It was found that the LIN primer set was suitably selective to amplify the *dhaA* gene of *P. pavonacae* 170 and *R. erythropolis* NCIMB13064 and not the *dhlA* gene of *X. autotrophicus* GJ10 and *A. aquaticus* strains AD25 and AD27 while the DHM primer amplified the *dhlA* gene of *X. autotrophicus* GJ10 and *A. aquaticus* strains AD25 and AD27 and not the *dhaA* gene of *P. pavonacae* 170 and *R. erythropolis* NCIMB13064. The production of a 450bp amplicon in the South African isolates indicated that that these isolates may posses a hydrolytic dehalogenase gene similar to the *dhlA* gene of *X. autotrophicus* GJ10 (Janssen *et al.*, 1989) and *A. aquaticus* strains AD25 and AD27 (van den Wijngaard *et al.*, 1992) or *dhm* gene of *Mycobacterium tuberculosis* H37Rv (Jesenska *et al.*, 2000).

The use of primers to amplify part of the 16S rDNA region of bacteria for identification purposes has become a common practice and is used extensively (Marchesi *et al*., 1998). BLAST analysis of the 16S rDNA sequence between nucleotides 63 and 1387 showed that all isolates belong to the genus *Ancylobacter* as they showed more than 99% sequence similarity but were different from the previously characterized *A. aquaticus* AD27 (van den Wijngaard *et al*., 1992). Multiple sequence alignment of the five South African isolates as well as *A. aquaticus* AD27 using the DNA analysis software package DNAMAN (Lynon Biosoft) indicated that all isolates had base substitutions at various points along the gene thus indicating that the five isolates are not identical to each other. Gram staining indicated that the bacterial isolates were Gram negative and had a horse-shoe shape which is typical of the genus *Ancylobacter*. Biochemical tests indicated a carbohydrate utilization profile similar to the previously characterized isolates of *Ancylobacter* (van den Wijngaard *et al*., 1992)

The successful isolation and preliminary identification of bacterial isolates capable of DCA degradation from waste water treatment facilities indicates that these facilities possess an enormous reservoir of unexplored microbes. These isolates utilize DCA as a growth substrate and thus have the potential to be used in future bioremediation strategies.

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CHAPTER THREE

ORGANISATION OF 1,2-DICHLOROETHANE (DCA) DEGRADING GENES

3.1 INTRODUCTION

Several bacterial species have thus far been identified that are capable of the complete aerobic mineralization of DCA (Stucki *et al*., 1983; Janssen *et al*., 1984; van den Wijngaard *et al*., 1992; Hage and Hartmans, 1999, Song *et al*., 2004). Coincidentally, all of these isolates also possess the same gene involved in the initial step of hydrolytic degradation of DCA (Janssen *et al*., 1989; van den Wijngaard *et al*., 1992; Song *et al*., 2004). The complete DCA degradation route was first reported by Janssen *et al*. (1984) and it would appear that the other isolates may degrade DCA via a similar route.

| A | B | C | D | E |
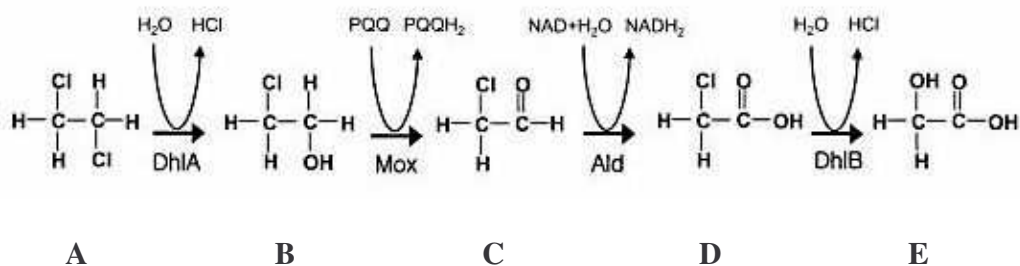
Fig. 3.1:   Proposed catabolic pathway of DCA degradation (A) DCA; (B) 2-chloroethanol; (C) 2-chloroacetaldehyde; (D) monochloroacetic acid; (E) glycolic acid; DhlA, haloalkane dehalogenase; Mox, alcohol dehydrogenase; Ald, aldehyde dehydrogenase; DhlB, haloacetate dehalogenase (Song *et al*., 2004).

Figure 3.1 illustrates the proposed catabolic route involved in DCA degradation by *X. autotrophicus* GJ10 (Janssen *et al*., 1985). The first gene involved (*dhlA*) with a corresponding nucleotide length of 933bp codes for the first enzyme involved in DCA catabolism, a hydrolytic dehalogenase (Janssen *et al*., 1989). This gene was later found to be located on a large plasmid (pXAU1) (Tardif *et al*., 1991). This gene has also been identified in *Ancylobacter aquaticus* AD20, AD25 and AD27 (van den Wijngaard *et al*., 1992) and *Xanthobacter flavus* UE15 (Song *et al*., 2004). The hydrolytic conversion of DCA results in the production of highly toxic 2-chloroethanol (Janssen *et al*., 1985).

Toxic 2-chloroethanol is then oxidised by an aspecific (pyrrolo-quinoline quinine) PQQ-dependant alcohol dehydrogenase. This enzyme has been shown to be an inducible methanol dehydrogenase with high activity towards 2-chloroethanol when isolated from *X. autotrophicus* GJ10 cultures grown on 2-chloroethanol as a carbon source. The enzyme consists of a 60kDa polypeptide that is associated with a 10 kDa polypeptide and contained (PQQ) as a prosthetic group. The oxidation of 2-chloroethanol results in the formation of another toxic intermediate, 2-chloroacetaldehyde (Janssen *et al*., 1987).

Janssen *et al*. (1985) first described that chloroacetaldehyde (CAA) is converted to a haloalkanoate by an aldehyde dehydrogenase in *X. autotrophicus* GJ10. It was later shown that at least three different aldehyde dehydrogenases may be produced by *X. autotrophicus* GJ10 of which one may be plasmid encoded. It also appeared that the chromosomally encoded aldehyde dehydrogenases may also be required for the efficient NAD-dependent oxidation of chloroacetaldehyde (van der Ploeg *et al*., 1994). The

plasmid encoded and chromosomally encoded homologs were later cloned, sequenced and expressed in *E. coli*. Based on the DNA-predicted amino acid sequences the plasmid encoded CAA dehydrogenase (AldA) was 505 amino acids in length while the chromosomally encoded homolog (AldB) was 506 amino acids in length. Both of the CAA dehydrogenases share an 84% amino acid identity (Bergeron *et al*., 1998).

The final step in the complete mineralization of DCA by *X. autotrophicus* GJ10 involves the conversion of monochloroacetic acid to glycolate by a haloacid dehalogenase which can then be used in the cell's central metabolic pathways (Janssen *et al*., 1985). The haloacid dehalogenase (DhlB) from *X. autotrophicus* GJ10 has been cloned and sequenced. The enzyme is 253 amino acids in length and has been shown to have activity against several different haloacids (van der Ploeg *et al*., 1991). Several different α-halocarboxylic acid (αHA) dehalogenase genes have been characterized and have been classified into two groups (group I and group II *deh* genes) based on their deduced amino acid sequences. It was shown that the two families are evolutionarily unrelated and together represent almost all of the αHA *deh* genes (Hill *et al*., 1999).

Recently, *X. flavus* UE15 was isolated from wastewater and shown to degrade DCA as well as several other chloroaliphatics by hydrolytic dechlorination under aerobic conditions (Song *et al*., 2004). It was found that the gene encoding the DCA dechlorinase was 933bp in length and deduced amino acid sequence exhibited 100% sequence identity with the corresponding enzyme from *X. autotrophicus* GJ10. Both organisms also possessed a transposase with 99% identity at the amino acid level. In *X.*

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flavus UE15 the transposase lies upstream to the *dhlA* gene (Fig. 3.2) whereas in *X. autotrophicus* the transposase lies upstream to the *dhlB* gene (Fig. 3.2). It has been shown that the *dhlA* and *dhlB* genes were located in different chromosomal loci in *X. flavus* UE15 and *X. autotrophicus* GJ10 (Janssen *et al.*, 1989; van der Ploeg *et al.*, 1991; Song *et al.*, 2004).
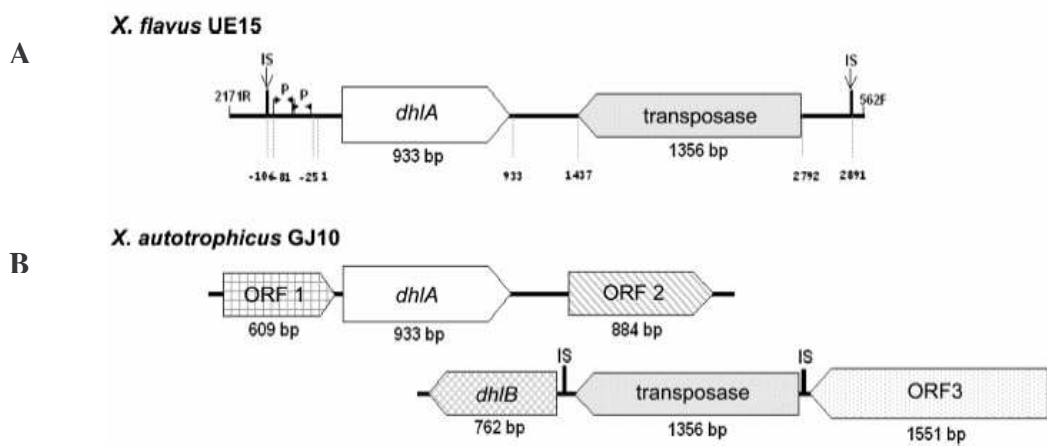
Fig. 3.2: Genetic organization of *dhlA* and transposase genes from *X. flavus* UE15 (**A**) and *X. autotrophicus* GJ10 (**B**). P: promoter; IS: target site for insertion element IS1247 (Song *et al.*, 2004).

The focus of this chapter was to amplify, clone and sequence the hydrolytic dehalogenase gene from the five South African isolates of *Ancylobacter*. Primers were also designed to amplify the haloacid dehalogenase gene (*dhlB*); the transposase (TPS) as well as the two aldehyde dehydrogenase gene homologs (*aldA* and *aldB*) from *X. autotrophicus* GJ10 in order to determine whether corresponding genes existed in the South African isolates. Southern blot analysis was also carried out using *X. autotrophicus* GJ10 gene probes in

order to determine whether these genes were within similar loci in both *X. autotrophicus* GJ10 as well as isolates of *Ancylobacter*. Using the primers designed for the *dhlA*, *dhlB* and transposase genes attempts were made to determine whether these genes had a similar organization compared to *X. autotrophicus* GJ10 and *X. flavus* UE15.

3.2   MATERIALS AND METHODS

3.2.1   Haloalkane degrading bacteria used in this study

Table 3.1 lists the haloalkane degrading bacteria used in this study. *X. autotrophicus* GJ10, *A. aquaticus* AD25 and AD27 were used as controls in this study.

Table 3.1:   Haloalkane degrading bacteria used in this study

Bacterial strain	Source
A. aquaticus DH2	This study
A. aquaticus DH5	This study
A. aquaticus DH12	This study
A. aquaticus UV5	This study
A. aquaticus UV6	This study
A. aquaticus AD25	D. B. Janssen*
A. aquaticus AD27	D. B. Janssen*
X. autotrophicus GJ10	D. B. Janssen*

* D. B. Janssen: Biochemical Laboratory, Biomolecular Sciences and Biotechnology Institute, University of Groningen, Groningen, The Netherlands

3.2.2 Maintenance and cultivation of bacterial strains

All working cultures were sub-cultured and maintained on LB agar (10g tryptone, 5g yeast extract, 5g NaCl and 12g bacteriological agar per litre) plates. LB broth (10g tryptone, 5g yeast extract and 5g NaCl per litre) cultures were used for the DNA isolations.

3.2.3 Analysis of genes involved in DCA degradation

3.2.3.1   PCR amplification of genes

PCR primers were designed based on the known sequences of the region flanking the hydrolytic dehalogenase gene (*dhlA*), aldehyde dehydrogenase genes (aldA and aldB), transposase gene and haloacetate dehalogenase gene (dhlB) from *X. autotrophicus* GJ10. Two homologs of the aldehyde dehydrogenase gene exist in *X. autotrophicus* GJ10 (*aldA* and *aldB*) which are plasmid encoded and chromosomally encoded, respectively. The transposase identified in *X. autotrophicus* GJ10 which lies upstream to the halocarboxlylic dehalogenase gene, *dhlB* has also been identified in *X. flavus* UE15 but it lies upstream to the hydrolytic dehalogenase gene, *dhlA* (Song *et al*., 2004). Primers indicated in Table 3.2 were synthesized by Inqaba Biotech and delivered in a lyophilized form. Total DNA was isolated using the QIAamp DNA Mini Kit (Qiagen) from all South African isolates as well as *X. autotrophicus* GJ10, *A. aquaticus* AD25 and AD27 and quantified using the GeneQuant, RNA/DNA Calculator (Pharmacia).

Table 3.2: Primer sequences of genes involved in DCA degradation

Gene	Primer name	Sequence of primer (5' to 3')
dhlA	dhl001F	GTCGTTGCTAGGTCACCCGAC
	dhl002R	CTCTATTCTGTCTCGGCAAAG
aldA	ALDAF	CAAGCCAGAAATCGCCATCAC
	ALDAR	CCCCAATGGGGAGAGGAAAGC
aldB	ALDBF	GGCGTGCGGTGCAGCTTCGAG
	ALDBR	CGGTGAAACGAAACGCGCGCG
transposase	TPS001F	TCATGCGCATAGCGGTGGCGC
	TPS002R	ATGGATCACCCAGAGAGTGCG
dhlB	DHLBF	CTGCGCGATGATGCGCAGGCC
	DHLBR	CCAATGGCCGCCGCCCATCTC

Stock solutions of primers were prepared by the addition of the appropriate amount of sterile de-ionised water to create a 1M stock while working stocks were at 100mM. Both primer stocks were stored at -20°C until required. The PCR mixtures (50µL) contained 10ng DNA, 100pmol of each of the appropriate primers, 100µM of each of the deoxynucleoside triphosphates (dNTPs), 1 x Super-therm *Taq* polymerase buffer and 0.5U Super-therm *Taq* polymerase (Southern Cross Biotech) finally brought to volume with sterile double distilled water. PCR was performed using the PE Applied Biosystems GeneAmp PCR System 9700, programmed to perform an initial denaturation at 94°C for 5 min and 30 cycles consisting of 94°C for 2 min, 58°C for 1 min and 72°C for 2 min

followed by a final extension step of 72˚C for 5 min. PCR products were separated on a 2% agarose gel, stained with ethidium bromide and documented using the Chemi-Genius Bio Imaging System (Syngene).

3.2.3.2 Restriction and electrophoresis of genomic DNA

Total DNA was isolated using the QIAamp DNA Mini Kit (Qiagen) and quantified using the GeneQuant, RNA/DNA Calculator (Pharmacia). Total DNA was restricted with *Eco*RI, *Pst*I, *Sma*I and *Bcl*I (Roche) as follows: 1µg DNA, 1U restriction endonuclease, 1 X restriction endonuclease buffer and the total volume was brought up to 10µl. Reactions were incubated at 37ºC for 2-3hours prior to electrophoresis on a 1% TAE agarose gel. The gels were stained with ethidium bromide for 30min and documented using the Chemi-Genius Bio Imaging System (Syngene).

3.2.3.3 Southern hybridization

Restricted DNA from the agarose gels were transferred to charged nylon membranes under neutral conditions by capillary action according to Ausubel *et al*. (1989). The use of charged nylon membranes does not require UV fixing. Membranes were stored in polypropylene bags after drying until required. Individual probes were prepared by labeling the cleaned *dhlA*, *aldB*, transposase and *dhlB* PCR products from *X. autotrophicus* GJ10 with digoxygenin according to the DIG High Prime Labeling and Detection System (Roche). Hybridisation, stringency washes and colorimetric detection were carried out using the DIG High Prime Labeling and Detection System (Roche) according to the manufactures instructions.

3.2.4　　　　　Cloning and sequencing of the *dhlA* gene

Following confirmation that the PCR products obtained were of the correct size, products were purified using the Qiaquick PCR Purification Kit (Qiagen). Cleaned products were ligated to the pGEM-T Easy Vector System I (Promega) and electro-transformed into electro-competent *E. coli* DH5α. Transformants were selected on LB agar (10g tryptone, 5g yeast extract, 5g NaCl and 12g bacteriological agar per litre) plates containing 100µg/ml ampicillin. Positive transformant colonies were grown overnight in 3ml LB broth (10g tryptone, 5g yeast extract, 5g NaCl) containing 100µg/ml ampicillin. Plasmid DNA was isolated using the High Pure Plasmid Isolation Kit (Roche) and sequenced from both directions using the T7 and SP6 primers by Inqaba Biotech. Sequences were then edited and aligned using the DNAMAN DNA analysis software (Lynon Biosoft). Sequences were aligned to each other as well as to the *dhlA* genes of *X. autotrophicus* GJ10 and *X. flavus* UE15.

3.2.5　Comparison of gene organization

Both *X. flavus* UE15 and *X. autotrophicus* GJ10 show different organizations of the hydrolytic dehalogenase gene, *dhlA*. No previous studies have analysed the organization of these genes in *A. aquaticus* isolates. In order to determine the organisation of the *dhlA*, *dhlB* and transposase gene in *A. aquaticus* different combinations of the different primers were used. The different combinations are *dhlA*F/TPSF and dhlBR/TPSF to determine whether these genes lay adjacent to each other.

3.3   RESULTS

3.3.1   Analysis of the hydrolytic dehalogenase gene, *dhlA*

The PCR primers designed to amplify the *dhlA* gene of *X. autotrophicus* GJ10 based on sequences flanking the *dhlA* gene were able to amplify this region in *X. autotrophicus* GJ10 (Fig. 3.3, lane 8). A region of similar size was also amplified in all of the isolates of *Ancylobacter* (Fig. 3.3, lanes 1 to 7) which may indicate that this region is conserved in *X. autotrophicus* GJ10 as well as isolates of *Ancylobacter*.

3.3.1.1   PCR amplification
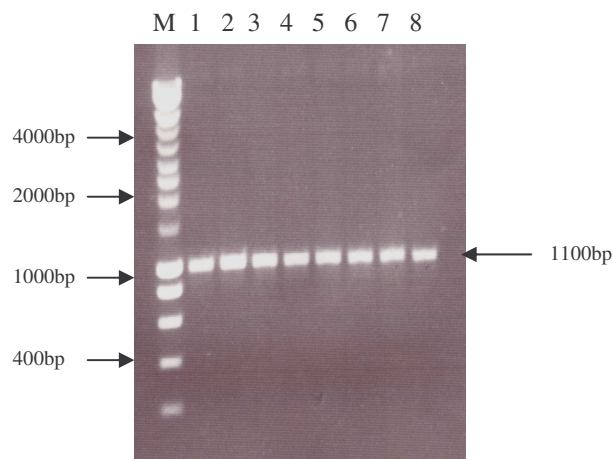
Fig. 3.3:   PCR amplification of the *dhlA* gene. M: Smart ladder; Lane1: DH2; lane 2: DH5; lane 3: DH12; lane 4: UV5; lane 5: UV6; lane 6: *A. aquaticus* AD25, lane 7: A. *aquaticus* AD27; lane 8: *X. autotrophicus* GJ10.

3.3.1.2   Cloning and sequencing of the *dhlA* gene

The region containing the *dhlA* gene that was amplified using the primers described in Section 3.2.3.1 was successfully cloned into the pGEM-T Easy Vector System I (Promega). Restriction of plasmid DNA preparations from positive transformants with *Eco*RI (Fig. 3.4) indicated that all clones contained the correct size insert. Clones were sequenced by Inqaba Biotech and edited sequences were aligned to the *dhlA* gene of *X. autotrophicus* GJ10 and *X. flavus* UE15 (Appendix B). The *dhlA* gene was found to be identical in all isolates as indicated in Appendix A.
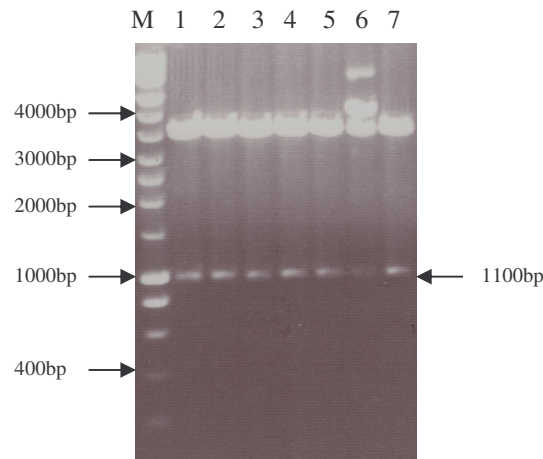
Fig. 3.4:   *Eco*RI digest of the cloned PCR amplified *dhlA* gene. M: Smart ladder; Lane1: DH2; lane 2: DH5; lane 3: DH12; lane 4: UV5; lane 5: UV6; lane 6: *A. aquaticus* AD25, lane 7: *A. aquaticus* AD27.

3.3.1.3   Southern hybridization of the *dhlA* gene

Fig. 3.5 shows that all of the isolates produce a signal corresponding to approximately 8.3 kb when using the *dhlA* gene as a probe. This indicates that this large region within the genomes of both *X. autotrophicus* GJ10 and isolates of *Ancylobacter* may be conserved.

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Figure 3.6 indicates the signals obtained when the *dhlA* probe was used against *Bcl*I digested genomic DNA. *Bcl*I restricts the *dhl*A gene of *X. autotrophicus* GJ10 once and the presence of two signals (lane 8) corresponds to the expected result. Two signals are also observed for *A. aquaticus* AD25 (lane 6) but appear to fractionally differ in size compared to the signals obtained for *X. autotrophicus* GJ10. Three signals are observed for *A. aquaticus* AD27 (lane 7) as well as the five South African isolates (lanes 1 to 5) that are of corresponding size. This may indicate that the regions upstream and down stream to the *dhlA* gene may be similar in *A. aquaticus* AD27 and the five South African isolates of *Ancylobacter* but different to *A. aquaticus* AD25 and *X. autotrophicus* GJ10. The presence of an additional signal for *A. aquaticus* AD27 and the five South African isolates may also indicate the presence of another region within the genome of these organisms that show similarity to the *dhlA* gene of *X. autotrophicus* GJ10.
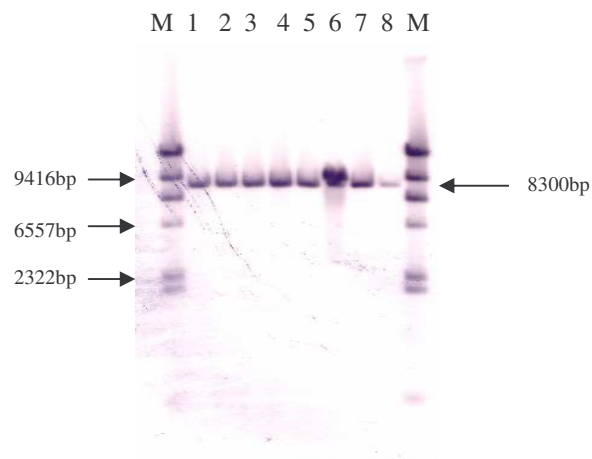
Fig. 3.5:   Hybridization of *dhlA* labeled probe to *Eco*RI digested total DNA. M: Marker II; Lane1: DH2; lane 2: DH5; lane 3: DH12; lane 4: UV5; lane 5: UV6; lane 6: *A. aquaticus* AD25, lane 7: A. *aquaticus* AD27; lane 8: *X. autotrophicus* GJ10 and M: Marker II.
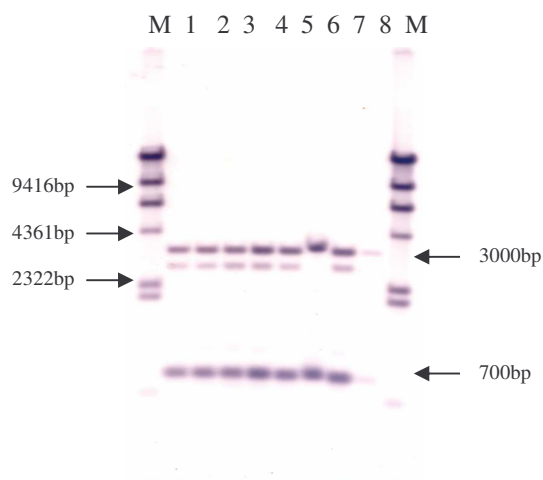
Fig. 3.6: Hybridization of *dhlA* labeled probe to *Bcl*I digested total DNA. M: Marker II; Lane1: DH2; lane 2: DH5; lane 3: DH12; lane 4: UV5; lane 5: UV6; lane 6: *A. aquaticus* AD25, lane 7: A. *aquaticus* AD27; lane 8: *X. autotrophicus* GJ10 and M: Marker II.

3.3.2 Analysis of the aldehyde dehydrogenase genes, *aldA* and *aldB*

Primers designed to amplify the *aldA* gene did not produce any amplicons even after several attempts. This may be due to incorrect primer synthesis or errors in the sequence present in the NCBI database. The primers designed to amplify the *aldB* gene of *X. autotrophicus* GJ10 produced the correct size product (Fig. 3.7, lane 8) but no similar sized products were observed in any of the other isolates. This indicates that the isolates of *Ancylobacter* do not possess the *aldB* gene. A smaller product observed in all of the isolates except *A. aquaticus* AD25 may be due to non-specific primer binding.

Southern hybridization using the PCR amplified *aldB* gene of *X. autotrophicus* GJ10 as a probe against *Sma*I digested DNA from all isolates showed the presence of multiple signals for all isolates (Fig. 3.8). The *aldA* gene contains a single *Sma*I restriction site

while there are two *Sma*I restriction sites flanking the *aldB* gene of *X. autotrophicus* GJ10 (Bergeron *et al*., 1998). Two signals were observed for *X. autotrophicus* GJ10, a lighter signal and a darker signal (Fig. 3.8, lane 8). The two signals observed for the South African isolates and *A. aquaticus* AD27 (Fig. 3.8, lanes1 to 5 and lane 7, respectively) correspond to fragments of lower molecular weight and appear to be of equal intensity. Three signals are observed for *A. aquaticus* AD25 (Fig. 3.8, lane 7), two of the signals are of similar size to the signals observed for the South African isolates and *A. aquaticus* AD27 but the third signal corresponds to a fragment larger than 10kb. This signal may be due to partially digested DNA or may actually be an aldehyde dehydrogenase homolog.

3.3.2.1   PCR amplification
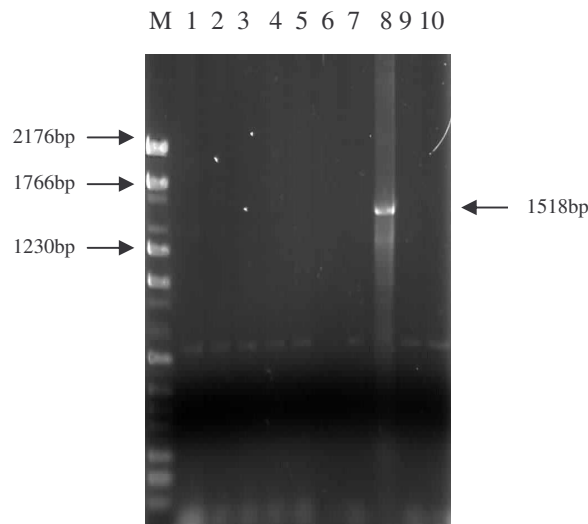
Fig. 3.7:   PCR amplification using the *aldB* gene. M: Marker VI; Lane1: DH2; lane 2: DH5; lane 3: DH12; lane 4: UV5; lane 5: UV6; lane 6: *A. aquaticus* AD25, lane 7: A. *aquaticus* AD27; lane 8: *X. autotrophicus* GJ10; lane 9: *R. erythropolis* NCIMB13064 and lane 10: *P .pavonacae* 170.

3.3.2.2 Southern hybridization of the *aldB* gene

M 1 2 3 4 5 6 7 8 M

23130bp ⟶

4361bp ⟶

2322bp ⟶

⟵ 3100bp

1700bp ⟶

⟵ 1900bp

1550bp ⟶
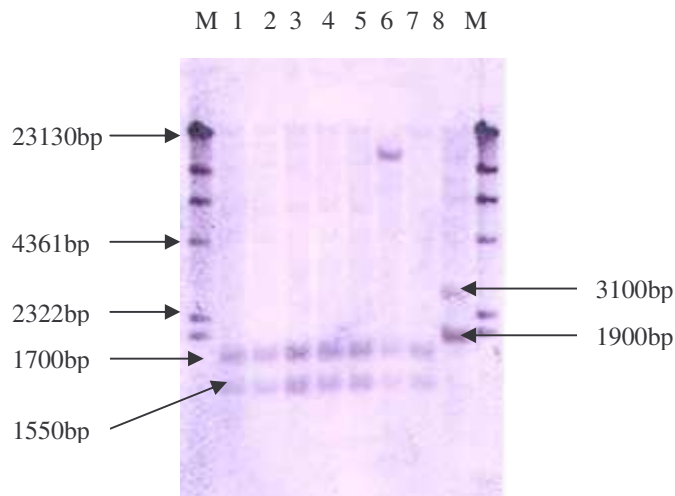
Fig. 3.8: Hybridization of *aldB* labeled probe to *Sma*I digested total DNA. M: Marker II; Lane1: DH2; lane 2: DH5; lane 3: DH12; lane 4: UV5; lane 5: UV6; lane 6: *A. aquaticus* AD25, lane 7: A. *aquaticus* AD27; lane 8: *X. autotrophicus* GJ10 and M: Marker II.

3.3.3 Analysis of the transposase gene

In Figure 3.9, PCR products can be observed for *Ancylobacter* isolates DH12 (lane 3); UV5 (lane 4); *A. aquaticus* AD27 (lane 7) and *X. autotrophicus* GJ10 (lane 8). The primers used were designed based on sequences flanking the transposase gene of *X. autotrophicus* GJ10. These products are of similar size which may indicate that a similar transposase is present. The use of the transposase gene which conatins a single *Eco*RI restriction site as a probe against *Eco*RI digested total DNA from all isolates indicated the presence of multiple signals for some of the isolates and single signals for others (Fig. 3.10). *X. autotrophicus* GJ10 (Fig. 3.10, lane 8) produced more than one signal indicating the possible presence of more than one transposase homolog. Isolates DH2 (lane 1), DH5 (lane 2) and UV6 (lane 5) produced single signals of similar size indicating

the possible presence of a single homolog present within the same genomic locus. This signal corresponding to a genomic fragment possibly larger than 10kb can be observed in all isolates of *Ancylobacter* except *A. aquaticus* AD25. The presence of multiple signals in *Ancylobacter* isolates DH12 and UV5 as well as *A. aquaticus* AD25 and AD27 may indicate the presence of multiple homologs of the transposase within these isolates.

The restriction endonuclease *Bcl*I recognises two restriction sites within the transposase of *X. autotrophicus* GJ10 and the presence of multiple signals when the transposase gene was used as a probe against *Bcl*I digested total DNA further indicates the presence of homologs (Fig. 3.11). The signals observed for isolates DH2 (lane 1), DH5 (lane 2) and UV6 (lane 5) once again appear similar indicating that the genomic region containing the transposase in these isolates is conserved.

3.3.3.1 PCR amplification of the transposase gene
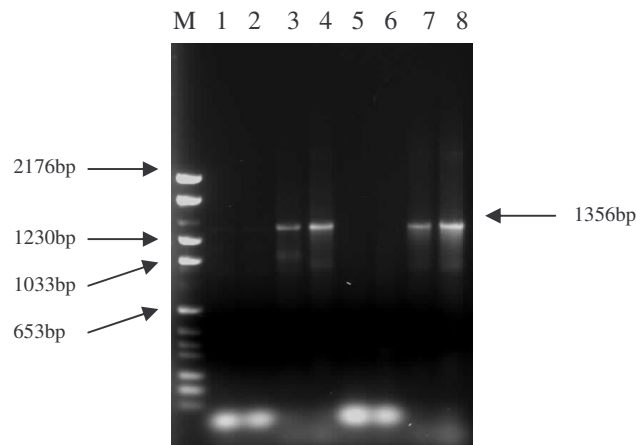
Fig. 3.9: PCR amplification of the transposase gene. M: Marker VI; Lane1: DH2; lane 2: DH5; lane 3: DH12; lane 4: UV5; lane 5: UV6; lane 6: *A. aquaticus* AD25, lane 7: *A. aquaticus* AD27; lane 8: *X. autotrophicus* GJ10.

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3.3.3.2   Southern hybridization of the transposase gene
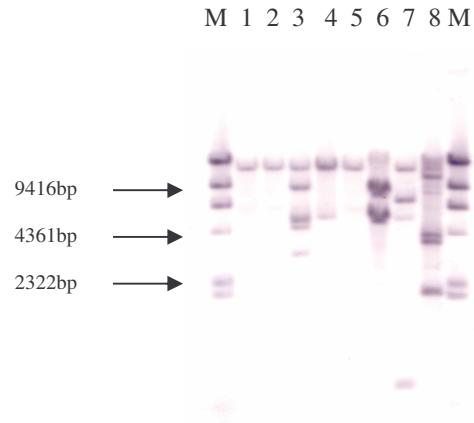
Fig. 3.10:   Hybridization of the labeled transposase gene probe to *Eco*RI digested total DNA. M: Marker II; Lane1: DH2; lane 2: DH5; lane 3: DH12; lane 4: UV5; lane 5: UV6; lane 6: *A. aquaticus* AD25, lane 7: A. *aquaticus* AD27; lane 8: *X. autotrophicus* GJ10 and M: Marker II.
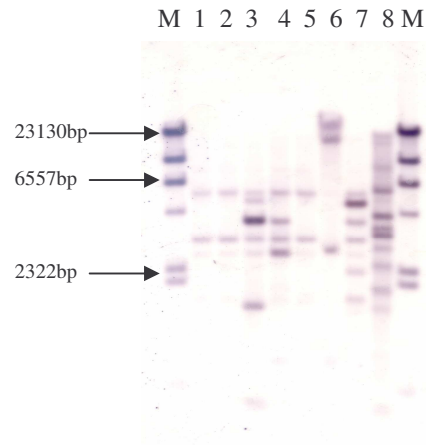
Fig. 3.11:   Hybridization of the labeled transposase gene probe to *Bcl*I digested total DNA. M: Marker II; Lane1: DH2; lane 2: DH5; lane 3: DH12; lane 4: UV5; lane 5: UV6; lane 6: *A. aquaticus* AD25, lane 7: A. *aquaticus* AD27; lane 8: *X. autotrophicus* GJ10 and M: Marker II.

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3.3.4   Analysis of the haloacetate dehalogenase gene, *dhlB*

The primer set designed to amplify the *dhlB* gene of *X. autotrophicus* GJ10 based on sequences flanking the gene did not result in the production of PCR products for any of the isolates besides *X. autotrophicus* GJ10 (Lane 8, Fig. 3.12).   Faint non-specific products were observed for isolates DH5 (lane 2), DH12 (lane 3 and UV5 (lane 4).   This indicates that the region flanking the *dhlB* gene in *X. autotrophicus* GJ10 is not conserved in isolates of *Ancylobacter*.

Fig. 3.13 shows hydribidisation of the *dhlB* labeled probe against *Pst*I digested DNA which does not restrict the *dhlB* gene.   The presence of multiple signals (lane 8) indicates that *X. autotrophicus* GJ10 may contain multiple homologs of the *dhlB* gene.   Signals were also obtained for isolates DH12 (lane 3), UV5 (lane 4) as well as *A. aquaticus* AD27 (lane 7).   The signals observed for isolate UV5 (lane 4) and *A. aquaticus* AD27 (lane 7) are of a similar size indicating that these isolates may contain *dhlB* homologs of a similar size.   This signal is also observed in lane 3 (isolate DH12) but an additional higher molecular weight signal is also observed indicating that a different homolog may be present in this isolate.

When *Bcl*I digested DNA was used as the template for the *dhlB* gene probe, two signals should be observed for *X. autotrophicus* GJ10 which restricts the *dhlB* gene once.   The presence of multiple signals once again indicates that more than one *dhlB* homolog may be present in *X. autotrophicus* GJ10 (Fig. 3.14, lane 8).   Signals are once again observed for isolates DH12 (lane 3), UV5 (lane 4) as well as *A. aquaticus* AD27 (lane 7).   A

common 6.5kb signal is observed for all three isolates but an additional 9.4kb signal can be observed for isolate DH12 (lane 3) which may once again indicate that the homolog present in DH12 is different.

3.3.4.1 PCR amplification of the *dhlB* gene
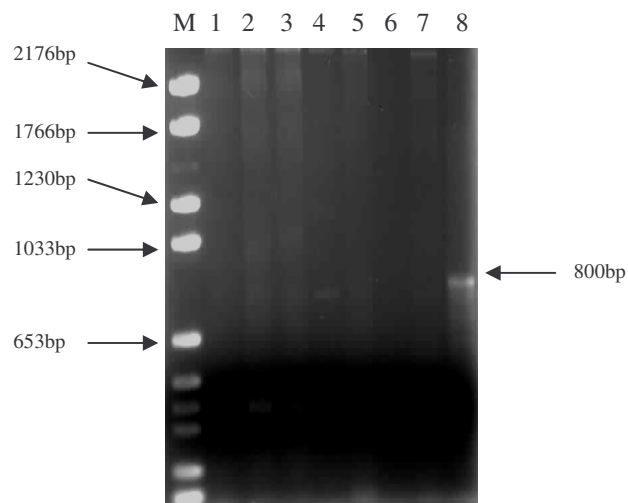
Fig. 3.12: PCR amplification of the *dhlB* gene. M: Marker VI; Lane1: DH2; lane 2: DH5; lane 3: DH12; lane 4: UV5; lane 5: UV6; lane 6: *A. aquaticus* AD25, lane 7: A. *aquaticus* AD27; lane 8: *X. autotrophicus* GJ10.

3.3.4.2 Southern hybridization of the *dhlB* gene
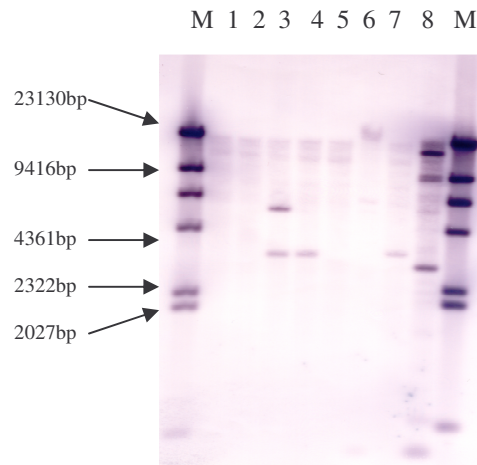
Fig. 3.13: Hybridization of *dhlB* labeled probe to *Pst*I digested total DNA. M: Marker II; Lane1: DH2; lane 2: DH5; lane 3: DH12; lane 4: UV5; lane 5: UV6; lane 6: *A. aquaticus* AD25, lane 7: A. *aquaticus* AD27; lane 8: *X. autotrophicus* GJ10 and M: Marker II.
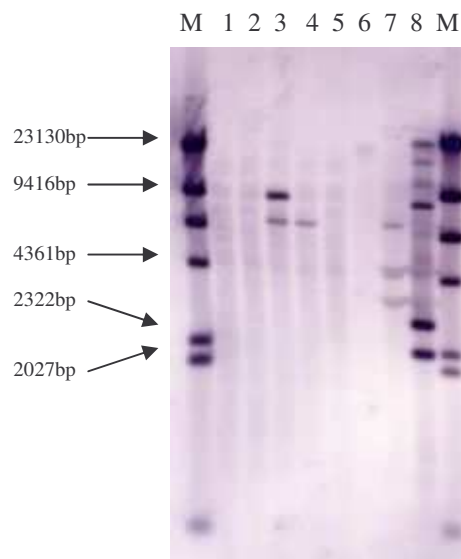
Fig. 3.14: Hybridization of *dhlB* labeled probe to *Bcl*I digested total DNA. M: Marker II; Lane1: DH2; lane 2: DH5; lane 3: DH12; lane 4: UV5; lane 5: UV6; lane 6: *A. aquaticus* AD25, lane 7: A. *aquaticus* AD27; lane 8: *X. autotrophicus* GJ10 and M: Marker II.

3.3.5 Comparison of gene organization

In order to determine whether the transposase lies adjacent to the *dhlA* (as observed in *X. flavus* UE15) or *dhlB* (as in *X. autotrophicus* GJ10), different primer combinations were used. The *dhlA*/TPSF and *dhlB*/TPSF primer sets did not result in any amplification of these regions. This may be due to incorrect orientation of the primers or the inability of *Taq* DNA polymerase to amplify a large region separating the genes.

3.4 DISCUSSION

The previous Chapter described the isolation and preliminary characterization of the five South African isolates of *Ancylobacter* as well as the possession of a hydrolytic dehalogenase that may be related to *linB* gene of *S. paucimobilis* UT26 or the *dhlA* gene of *X. autotrophicus* GJ10 and *Ancylobacter aquaticus* AD25 and AD27. In this chapter an analysis is made of all of the genes that may be involved in the degradation of DCA. A comparison of the possible loci of these genes is also made in order to compare them to previously characterized 1,2-dichloroethane degraders. Identification of possible homologs was carried out by Southern hydridisation.

Primers designed for the amplification of the region flanking the *dhlA* gene of *X. autotrophicus* GJ10 based on sequences present within the NCBI database were successfully able to amplify the gene from the South African isolates and two other *A. aquaticus* isolates as well as the control organism *X. autotrophicus* GJ10 (Fig. 3.2). This

indicates that the regions directly flanking the *dhlA* gene of *X. autotrophicus* GJ10 are conserved in the five South African isolates of *Ancylobacter* as well as two previously characterized isolates.

The *dhlA* PCR products of the five South African isolates as well as *A. aquaticus* AD25 and AD27 were successfully cloned into pGEM-T Easy PCR Cloning vector (Promega) and sequenced by Inqaba Biotech. The sequence information was edited using the DNAMAN sequence analysis software (Lynon Biosoft) and compared to sequences present within the BLAST database. It was found that the *dhlA* genes of all South African isolates were 933bp in length and shared a 100% sequence identity with the hydrolytic dehalogenases of *X. autotrophicus* GJ10 (Janssen *et al.*, 1989) and *X. flavus* UE15 (Song *et al.*, 2004). NCBI accession numbers M26950 and AY561847, respectively.

Janssen *et al.* (1989) cloned and sequenced the *dhlA* gene of *X. autotrophicus* GJ10 from an 8.3kb *Eco*RI fragment of genomic DNA. A digoxygenin labeled *dhlA* PCR product hybridized to a common 8.3kb region in all South African isolates, two *Ancylobacter* isolates and *X. autotrophicus* GJ10 when total DNA was restricted with *Eco*RI (Fig. 3.5). This indicates that the region containing the *dhlA* gene is conserved in both *Ancylobacter* sp. and *X. autotrophicus* GJ10. In order to investigate whether the upstream and down stream regions are conserved total DNA was restricted with *Bcl*I which has a single restriction site within the *dhlA* gene. Hybridisation with a labeled *dhlA* probe indicated that the five South African isolates and *A. aquaticus* AD27 had common upstream and

downstream regions (Fig. 3.6). The signal observed for *A. aquaticus* AD25 is different from the others indicating that this strain has either a different upstream or downstream region. The signal obtained for *X. autotrophicus* GJ10 was too weak to conclusively determine whether it is similar or different from the *Ancylobacter* isolates. The presence of an additional signal for *A. aquaticus* AD27 and the five South African isolates may indicate the presence another region within the genome of these organisms that show similarity to *dhlA* gene of *X. autotrophicus* GJ10.

The metabolism of 1,2-DCA proceeds via 2-chloroethanol to chloroacetaldehyde by an inducible NAD-dependent chloroacetaldehyde dehydrogenase which is a reactive and potentially toxic intermediate (Janssen *et al*., 1987). It was found that *X. autotrophicus* GJ10 may actually possess at least three aldehyde dehydrogenases of which one may be plasmid encoded (van der Ploeg *et al*., 1994). Two chloroacetaldehyde dehydrogenases were later cloned and sequenced by Bergeron *et al*. (1998). The two aldehyde dehydrogenase genes, *aldA* a plasmid located aldehyde dehydrogenase and *aldB*, a chromosomally encoded aldehyde dehydrogenase had predicted amino acid sequence sizes of 505aa and 506aa, respectively and were 84% identical (Bergeron *et al*., 1998).

Primers were designed based on sequences of the regions flanking the two aldehyde dehydrogenase genes which were acquired from the NCBI database. Unfortunately, the primers that were designed to amplify *aldA* gene did not produce any amplicons. Possible reasons for this may include errors during the synthesis of the primers or possibly errors in the NCBI database sequence. Primers that were designed to amplify

the *aldB* in *X. autotrophicus* GJ10 produced the correct size product of approximately 1550bp but did not produce any products for the other organisms including *R. erythropolis* NCIMB13064 and *P. pavonacae* 170. A smaller insignificant PCR product was produced by the other organisms but not *A. aquaticus* AD25. This may indicate that the other organisms possess an aldehyde dehydrogenase gene that is different from gene possessed by *X. autotrophicus* GJ10.

The *aldB* PCR product of *X. autotrophicus* GJ10 was labeled with digoxygenin and then used as a probe against *Sma*I digested DNA blotted onto a nylon membrane. The results obtained for *X. autotrophicus* GJ10 (Fig. 3.8) are consistent with the description given by Bergeron *et al*. (1998). There is one *Sma*I restriction site within the *aldA* gene and there are two *Sma*I restriction sites flanking the *aldB* gene and with these genes being 84% identical there should ideally be three signals. This does not occur as the region downstream of the *Sma*I restriction site within *aldA* hybridizes at the same point as the *aldB* region producing a single signal (Fig. 3.8). This is observed as the much darker signal observed in the 2000bp region.

Two signals are observed for the five South African isolates as well as *A. aquaticus* AD27 which correspond to a smaller fragment than the signals obtained for *X. autotrophicus* GJ10. This indicates that the *Ancylobacter* isolates have one or two different aldehyde dehydrogenase genes compared to *X. autotrophicus* GJ10. The presence of signals indicates that the aldehyde dehydrogenases may be related at the sequence level. The *A. aquaticus* AD25 isolate produced an additional signal which is

larger than 10kb. This signal may be produced due to unrestricted DNA but the observation as a distinct band may indicate sequence homology to a *Sma*I fragment around the 10kb region.

Primers were designed based on sequences of the regions flanking the transposase gene of *X. autotrophicus* GJ10 in order to determine if the *Ancylobacter* isolates possessed a similar transposase. Distinct products were obtained for isolate DH12 and X. *autotrophicus* GJ10 while faint products were obtained for isolate UV5 and *A. aquaticus* AD27 (Fig. 3.9). The presence of PCR products indicates that these isolates may possess an identical or closely related transposase genes.

The use of the transposase gene PCR product as a digoxygenin labeled probe against blotted *Eco*RI digested DNA revealed the presence of single and multiple signals in the different isolates (Fig. 3.10). All of the South African isolates produced a common 15kb signal but isolates DH12 and UV5 produced distinct additional signals. The signals produced for *A. aquaticus* AD25 and AD27 as well as *X. autotrophicus* GJ10 were distinctly different. The single 15kb signal for isolates DH2, DH5 and UV6 indicates that these isolates may possess a single transposase gene within the same region. When DNA was digested with *Bcl*I and blotted it was found that isolates DH2, DH5 and UV6 once again produced similar signals (Fig. 3.11) indicating that these isolates possess the tansposase gene in a similar location. The presence of multiple signals for *X. autotrophicus* GJ10 when digested with *Eco*RI and *Bcl*I indicate the presence of multiple homologous transposase genes.

Primers designed to amplify the haloacid dehalogenase gene of *X. autotrophicus* GJ10 were based on sequences present within the NCBI database. These primers were able to successfully amplify the gene in *X. autotrophicus* GJ10 but in none of the other organisms (Fig.3.12). Although products corresponding to a lower molecular weight were obtained for isolates DH5 and DH12, these may be non specific products. *Pst*I has a single restriction site within the *dhlB* gene. When the *dhlB* PCR product of *X. autotrophicus* GJ10 was digoxygenin labeled and used as a probe against *Pst*I blotted genomic DNA three signals were obtained for *X. autotrophicus* GJ10 while signals were also obtained for isolates DH12, UV5 and *A. aquaticus* AD27 (Fig. 3.13). One of the signals were at the same point for both isolates while a second distinct signal was obtained for isolate DH12. Multiple signals were obtained for *X. autotrophicus* GJ10 when total DNA was digested with *Bcl*I when ideally only two signals should have been obtained (Fig.3.14). The presence of multiple signals for *X. autotrophicus* GJ10 may indicate the presence of more than one homolog of *dhlB*. The presence of different signals among the South African isolates and *A. aquaticus* AD27 indicate that these isolates may possess homologs to the *dhlB* gene. Attempts to determine whether the transposase gene lies adjacent to *dhlA* or *dhlB* genes were unsuccessful. This may not necessarily mean that these genes do not lie next to each other as the orientation in the design of the primers may have been incorrect or the amplification of such a large region may not be possible with the DNA polymerase used. Certain brands of DNA polymerase may not have the potential to amplify large regions of DNA that separate genes.

This chapter was able to show that the only genes that were amplifiable using the primers designed based on the sequences of *X. autotrophicus* GJ10 were the *dhlA* and the transposase in three isolates of *Ancylobacter*. When digoxygenin labeled probes were used it can be clearly seen that *dhlA* gene lies within a common locus in all isolates tested. It can also be seen that homologs to the others genes exist in some of the other isolates. The presence of homologs warrants further study.

3.5   REFERENCES

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CHAPTER FOUR

BIOCHEMICAL ACTIVITIES OF 1,2-DICHLOROETHANE (DCA)
DEGRADING BACTERIA

4.1   INTRODUCTION

At present halogenated aliphatic compounds constitute an important class of environmental pollutants within both terrestrial and aquatic ecosystems and various microorganisms have evolved that are able to degrade some of these compounds and use them as sole sources of carbon and energy (van Hylckama Vlieg *et al*., 2001). Microorganisms capable of degrading these chemicals have been isolated from contaminated sites and the genes and enzymes involved in their degradative pathways have been extensively studied (Fetzner and Lingens, 1994; Janssen *et al*., 2001). The catabolic pathway involved in the complete mineralisation of 1,2-dichloroethane was first identified by Janssen *et al*. (1985a) in *Xanthobacter autotrophicus* GJ10 (Fig. 4.1).
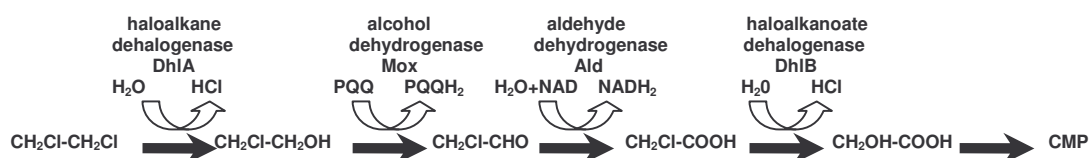
Fig. 4.1:   Catabolic route for the metabolism of DCA in *X. autotrophicus* GJ10, CMP (central metabolic pathway), (Adapted from Janssen *et al*., 1985a).

The first and usually most important step in the mineralization of chlorinated aliphatic compounds is known to be dehalogenation. Enzymes that cleave the carbon-halogen bond of haloaliphatics are generally referred to as dehalogenases and these may function either aerobically or anaerobically (Fetzner and Lingens, 1994). Thus far, numerous haloalkane dehalogenase homologues have been detected in both haloalkane degraders as well as in strains that are not known to degrade halogenated compounds (Table 4.1). The roles of the haloalkane dehalogenase homologues in the latter strains are not known.

Table 4.1: Haloalkane dehalogenases and putative haloalkane dehalogenases (Jesenska *et al.*, 2002)

Organism	Strain	Gene	Protein	Accession no.	
				Gene (EMBL/GenBank/DDBJ)	**Protein (SWISS-PROT)**
Sphingomonas paucimobilis	UT26	*linB*	LinB	D14594	P51698
Mycobacterium tuberculosis	H37Rv	*rv2579*	Rv2579[a]	Z77724	Q50642
Mycobacterium tuberculosis	CDC1551	*mt2656*	Rv2579[a]	AE007099	Q50642
Mycobacterium bovis	MU11	*iso-rv2579*	Iso-Rv2579[a]	AJ243259	Q9XB14
Mycobacterium smegmatis	MC2_155	*aal17946*	Aal17946[a]	AY054120	AAL17946
Rhodococcus (formerly *Corynebacterium*) sp.	m15-3	*dhaA*	DhaA	NA	Q53042
Rhodococcus (formerly *Arthrobacter*) sp.	HA1	*dhaA*	DhaA	NA	Q53042
Rhodococcus (formerly *Acinetobacter*) sp.	GJ70	*dhaA*	DhaA	NA	Q53042
Rhodococcus sp.	TB2	*dhaA*	DhaA	NA	Q53042
Rhodococcus erythropolis	Y2	*dhaA*	DhaA	NA	Q53042
Rhodococcus rhodochrous	NCIMB13064	*dhaA*	DhaA	AF060871	Q53042
Pseudomonas pavonaceae	170	*dhaA*	DhaA	AJ250371	Q53042
Mycobacterium sp.	GP1	*dhaA*$_f$	DhaA$_f$	AJ012627	Q9ZER0
Mesorhizobium loti	MAFF303099	*mlr5354*	Mlr5354[a]	AP003006	Q98C03

Xylella fastidiosa	9A5C	*xf1965*	Xf1965[a]	AE004016	Q9PC20
Photobacterium profundum	SS9	*aal01057*	Aa101057[a]	AF409100	AAL01057
Caulobacter crescentus	CB15	*cc1175*	Cc1175[a]	AE005795	Q9A919
Mycobacterium tuberculosis	H37Rv	*rv2296*	Rv2296[a]	Z77163	Q50670
Mycobacterium tuberculosis	CDC1551	*mt2353*	Rv2296[a]	AE007077	Q50670
Mycobacterium avium	N85	*dhmA*	DhmA	AJ314789	CAC41377
Mycobacterium avium	104	*106*	DhmA	NA	CAC41377
Xanthobacter autotrophicus	GJ10	*dhlA*	DhlA	M26950	P22643
Xanthobacter autotrophicus	GJ11	*dhlA*	DhlA	NA	P22643
Ancylobacter aquaticus	AD20	*dhlA*	DhlA	NA	P22643
Ancylobacter aquaticus	AD25	*dhlA*	DhlA	NA	P22643

[a]**Putative haloalkane dehalogenases identified by sequence similarity.**
[b]**NA, not available.**
[c]**R. D. Fleischmann *et al*., unpublished data.**
[d]**A. Jesenská *et al*., unpublished data.**

The *X. autotrophicus* GJ10 dehalogenase gives it the ability to use a number of short chain aliphatic compounds as sole carbon and energy sources. This constitutive haloalkane dehalogenase (DhlA) catalyses the hydrolytic dehalogenation of halogenated $C_2 - C_4$ alkanes such as chlorinated, brominated and iodated compounds to the corresponding haloalcohols (Keuning *et al*., 1985). It has been found that the haloalkane dehalogenase catalyses the hydrolytic release of halide from haloalkanes, resulting in the replacement of the halogen substituent by a hydroxyl group (Janssen *et al*., 1985b). Other hydrolytic haloalkane dehalogenases belonging to the same family that have been extensively studied include LinB from *Sphingomonas paucimobilis* (Nagata *et al*., 1993) and DhaA from both *Rhodococcus erythropolis* NCIMB13064 [formerly *R. rhodochrous* NCIMB13064] (Poelarends *et al*., 2000) and *Pseudomonas pavonaceae* 170 [formerly *P.*

cichorii 170] (Poelarends *et al*., 1998). The hydrolytic dehalogenase of *X. autotrophicus* GJ10 (DhlA) is also conserved within the facultative methylotrophs *Ancylobacter aquaticus* AD20, AD25 and AD27 (van den Wijngaard *et al*., 1992). It has also been recently found that *X. flavus* UE15 possesses an identical hydrolytic dehalogenase (Song *et al*., 2004).

The oxidation of 2-chloroethanol to chloroacetate in *X. autrophicus* GJ10 is catalysed by two different dehydrogenases. It was found that the chloroethanol dehydrogenase was a pyrrolo-quinoline quinone containing alcohol dehydrogenase (Mox) and due to its broad substrate specificity was active with 2-chloroethanol. Several methylotrophic bacteria have been identified that produce quinoprotein alcohol dehydrogenases which require ammonia or amines for activity and are able to catalyse the oxidation of several primary alcohols (Janssen *et al*., 1987). It was also shown that 2-chloroethanol was converted to chloroacetaldehyde by a phenazine methosulfate-linked alcohol dehydrogenase by *A. aquaticus* AD25. In addition to 2-chloroethanol the inducible alcohol dehydrogenase of this isolate was able to convert 2-bromoethanol, methanol and ethanol (van den Wijngaard *et al*., 1992).

The highly reactive and potentially toxic intermediate, chloroacetaldehyde is then converted to monochloroacetic acid (Janssen *et al*., 1985b). An inducible NAD-dependent chloroacetaldehyde dehydrogenase that catalyses the conversion of chloroacetaldehyde to monochloroacetic acid in *X. autotrophicus* GJ10 was first identified by Janssen *et al*. (1987). It was later reported that *X. autotrophicus* GJ10

produced at least three different aldehyde dehydrogenases of which one was plasmid-encoded (van der Ploeg *et al*., 1994). Bergeron *et al*. (1998) later identified a linear plasmid-based chloroacetaldehyde dehydrogenase gene (*aldA*) and a chromosomal homolog (*aldB*) in *X. autotrophicus* GJ10. Although isolates of *A. aquaticus* have a similar degradative route to *X. autotrophicus* GJ10 (van den Wijngaard *et al*., 1992), the aldehyde dehydrogenase genes have not been identified.

X. autotrophicus GJ10 has been shown to convert monochloroacetic acid to glycolate which enters into the cell's central metabolic pathway enabling it to completely utilize DCA as a sole carbon and energy source (Janssen *et al*., 1985b). The haloacid dehalogenase gene (*dhlB*) has been cloned and sequenced (van der Ploeg *et al*., 1994). Characterisation of the enzyme has revealed that this enzyme catalyses the cleavage of carbon-halogen bonds through a nucleophilic substitution by water to yield free halide resulting in it being characterized as a hydrolytic dehalogenase.

This chapter focuses on determining whether the South African isolates of *Ancylobacter aquaticus* possess a similar degradative pathway as *X. autotrophicus* GJ10 and three previously identified isolates of *Ancylobacter aquaticus* using biochemical assays. Intermediates of the DCA degradative pathway of *X. autotrophicus* GJ10 were used as substrates for each of the assay procedures. Polyacrylamide gel electrophoresis (PAGE) was also carried out in order to determine whether any of the enzymes were over-expressed.

4.2   MATERIALS AND METHODS

4.2.1   Bacterial isolates

Table 4.2 lists the haloalkane degrading bacteria used in this study. *X. autotrophicus* GJ10, *A. aquaticus* AD25 and AD27 were used as controls in this study.

Table 4.2:   Haloalkane degrading bacteria used in this study

Bacterial strain	Source
A. aquaticus DH2	This study
A. aquaticus DH5	This study
A. aquaticus DH12	This study
A. aquaticus UV5	This study
A. aquaticus UV6	This study
A. aquaticus AD25	D. B. Janssen*
A. aquaticus AD27	D. B. Janssen*
X. autotrophicus GJ10	D. B. Janssen*

* D. B. Janssen: Biochemical Laboratory, Biomolecular Sciences and Biotechnology Institute, University of Groningen, Groningen, The Netherlands

4.2.2   Growth of bacterial cultures

The culture medium used was described previously (Section 2.2.3) except that the vitamin solution was replaced with 30mg/l yeast extract with the new medium being referred to as MMY (Janssen *et al*., 1985a). The carbon source (1,2-dichloroethane) was supplemented to a final concentration of 5mM. Batch cultures were grown in 250ml

serum bottles that were filled to one-third their volume and were closed gas tight with Teflon-lined screw caps to prevent evaporation of volatile substrates. Large volumes of cultures were grown (2l) at 30˚C with rotary shaking. The five South African isolates as well as *A. aquaticus* AD25, AD27 and *X. autotrophicus* GJ10 were grown to late log phase.

4.2.3   Preparation of crude cell lysates and protein quantification

Cells were harvested by centrifugation (10min at 10 000 x g) and re-suspended in 100ml TEM buffer (10mM Tris sulphate, 1mM β-mercaptoethanol and 1mM EDTA, pH7.5). Cells were pelleted by centrifugation (10min, 10 000 x g) and resuspended in 10ml TEM buffer. Bacterial cells were lysed by sonication. A crude extract was obtained by centrifugation at 4°C for 30min at 40 000 x g. The crude extract was stored at 4˚C to prevent degradation of proteins and subsequent loss in activity (van der Wijngaard *et al*., 1992).

Protein was quantified according to the Bradford Method (Bradford, 1976). A protein standard curve was constructed using varying concentrations of BSA (Bovine Serum Albumin) Fraction V (Roche Biochemicals) in 0.15M NaCl as standards. Based on the regression equation, the total protein within the lysate was determined.

4.2.4   Assay procedures

In order to determine whether South African isolates possessed a catabolic route that was similar to that of *X. autotrophicus* GJ10 and other *Ancylobacter* isolates (van den

Wijngaard *et al.*, 1992) various intermediates in the pathway were used as substrates. The assays used, not only determined whether the various reactions were catalysed but also determined the quantity of substrate utilized. All assays were performed in triplicate and the averages of triplicate assays are indicated.

4.2.4.1　　Haloalkane dehalogenase

The colorimetric method used for the detection and quantification of halides has been described by Bergmann and Sanik (1957). The method used for this assay is adapted from Janssen *et al.* (1985b) and Keuning *et al.* (1985). The assay is based on the insolubility of $HgCl_2$, which is formed and precipitates when Cl^- is mixed with Hg(SCN). This releases SCN^-, which forms a red complex with Fe^{2+}. The $Fe(SCN)_2$ complex thus formed is stable for at least 1hour and measured at 460nm. It should be noted that the calibration curve is not linear and the assay can only be used for samples containing 0.01 – 2mM of chloride. As a precaution samples should always be freshly prepared and to prevent evaporation of substrates, stocks should be made in tightly closed vials containing only a small volume gas phase.

To prepare calibration curves 0.5ml of standard solutions of 0 – 2mM of potassium halides in H_2O were mixed with 1.8ml diluted reagent I [0.25M $NH_4Fe(SO_4)_2$ in 9M HNO_3]. Reagent I was diluted eight fold with H_2O before use. Colour development was initiated by the addition of 0.2ml of reagent II (saturated solution of $Hg(SCN)_2$ in absolute ethanol) and mixing. Spectophotometric readings were taken at 460nm using a standard UV/VIS spectrophotometer (Pharmacia). Water was used as a blank. Data were

then introduced into a spreadsheet and regression analysis was performed to construct a calibration curve.

The substrate solution (30ml) containing 5mM DCA (Aldrich) in 50mM Tris.SO$_4$ (pH7.5) was prepared in a 30ml serum bottle with a Teflon lined screwcap and 3ml was aliquoted into 5 tubes. A separate set of 5 tubes was also prepared containing 1.8ml diluted halide reagent I. Reactions were started by adding 200μl of the crude cell lysate to the tubes containing the substrate solution which was placed into a circulating water bath at 30ºC. Samples were taken after 1min (t=0) by removing 500μl of the incubation mixture and adding it to the first tube containing reagent I followed by the addition 0.2ml of reagent II. Sampling was repeated in the same way after different time intervals and added to the different tubes containing reagent I, followed by the addition of reagent II. Readings were taken at 460nm using a standard UV/VIS spectrophotometer (Pharmacia) using water as a blank. To ensure accuracy, all extinctions were below 0.4 absorbance units. The amount of free halide and dehalogenase activities were determined from the halide standard curves and protein standard curves. One unit is defined as the amount of enzyme that produces 1μmol of product per min under the conditions used. It should be noted that 2moles of halide are produced per mole of substrate for DCA because this assay is used to measure both the hydrolytic dehalogenase and haloacetate hehalogenase activity (Janssen *et al*., 1987).

4.2.4.2   Alcohol dehydrogenase

In order to measure the haloalcohol dehydrogenase activity of the cell free extracts of the South African isolates of *Ancylobacter*, the 2-chloroethanol-dependant reduction of 2,6-dichloroindophenol (DCIP) was followed spectrophotometrically at 600nm in a coupled fashion with phenazine methosulphate (PMS) as an artificial electron acceptor (Janssen *et al*., 1987).

Stock solutions of the following reagents were prepared prior to spectrophotometric analysis: 0.5M KH_2PO_4, pH8.0; 10mM PMS; 10mM DCIP; 100mM NH_4Cl and 10mM NaCN. In a 1.5ml quartz cuvette each of the components were added to a final volume of 1ml and concentration of 83mM KH_2PO_4; 15mM NH_4Cl; 0.09 mM NaCN; 0.1mM DCIP; 0.22mM PMS and 5mM 2-chloroethanol or 2-bromoethanol (Aldrich). Crude cell lysate (100μl) was then added and the final volume brought up to 1ml with water and mixed by inversion. The spectrophotometer was set to autozero and the activity was measured in "timedrive" mode at 30ºC immediately after adding the substrate to the cuvette. The decrease in absorbance was measured at 600nm for 10min (ε_{DCIP} = 21.9 $mM^{-1}cm^{-1}$ at 600nm).

The activity was calculated by obtaining the initial slope of the absorbance (1 – 3min) and dividing the result by 21.9 $mM^{-1}cm^{-1}$ to get the change in concentration. This change in concentration was converted to activity by multiplying it by 10 to account for the amount of crude cell lysate in the cuvette (100μl in this case). This result was then converted to specific activity by dividing it by the protein concentration. The activity

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was corrected by subtracting the value for zero activity. One unit of enzyme activity is defined as 1μmol substrate converted per minute (Janssen *et al*., 1987).

4.2.4.3   Aldehyde dehydrogenase

Aldehyde dehydrogenase activity of the crude cell lysate was measured spectrophotmetrically at 340nm by following the 2-chloroacetaldehyde-dependent reduction of NAD+ to NADH (Janssen *et al*., 1987). The reaction mixture contained (in a final volume of 1ml), 100mM sodium pyrophosphate (pH8.75), 1mM NAD, 1mM dithiothreitol, 10mM β-mercaptoethanol, 5mM chloroacetaldehyde (Aldrich), and 100μl crude cell lysate. After the spectrophotometer was set to autozero, the activity was measured in timedrive at 30˚C. The decrease in absorbance was measured at 340nm for 10min (ε_{NAD+} = 6.22mM^{-1}cm^{-1} at 340nm). The activity was calculated by obtaining the initial slope of the absorbance (1 to 3min) and dividing the result by ε = 6.22mM^{-1}cm^{-1} to get the change in concentration. This change in concentration was converted to activity by multiplying it by 10 to account for the amount of crude cell lysate in the cuvette (100μl). This result was then converted to specific activity by dividing it by the protein concentration. The activity is corrected by subtracting the value for zero activity. One unit is defined as the activity catalyzing the formation of 1μmol NADH per minute (Janssen *et al*., 1987).

4.2.4.4   Chloroacetate dehalogenase

The assay used to determine chloroacetate dehalogenase activity was similar to the procedure used to determine hydrolytic dehalogenase activity except that 1,2-

dichloroethane was replaced with 5mM monochloroacetic acid (Aldrich). One unit was defined as the amount of enzyme that produces 1µmol of halide per min under the conditions used. It should be noted here that 1 mole of halide is produced per mole of substrate for monochloroacetic acid (Janssen *et al.*, 1987).

4.2.5 SDS-PAGE analysis

The comparison of the individual protein components was determined by SDS-PAGE on 10% (w/v) polyacrylamide according to the method of Laemmli (1970). Each well was loaded with 100µg/ml crude cell lysate in order to determine if the 37kDa hydrolytic dehalogenase was over-expressed in any of the isolates. The reference protein sizes were 205 kDa, 116 kDa, 97 kDa, 66 kDa, 55 kDa, 45 kDa, 36 kDa, 29 kDa, 24 kDa, 20 kDa, 16.2 kDa and 6.5kDa (Broad Range Marker – Amersham).

4.3 RESULTS

4.3.1 Protein quantification

Table 4.3 shows the absorbance at 595nm of a protein concentration range between 0.0025mg/ml to 0.5mg/ml using the Bradford method. These values were used to construct the linear standard curve (Fig. 4.2) from which the linear regression equation, $f(x) = 1.6968x+0.3622$ was generated. Crude cell lysates were obtained following cell disruption by sonication and the total amount of protein present in the crude cell lysates was calculated using the linear regression equation (Table 4.4). The amount of protein in crude cell lysates would later be used to determine the specific activity of different enzymes involved in the DCA catabolic pathway.

Table 4.3: Absorbance at 595nm of different BSA concentrations using the Bradford method

Protein concentration (mg/ml)	Absorbance (595nm)
0	0.342
0.0025	0.355
0.005	0.362
0.01	0.377
0.025	0.409
0.05	0.457
0.1	0.536
0.25	0.798
0.5	1.203
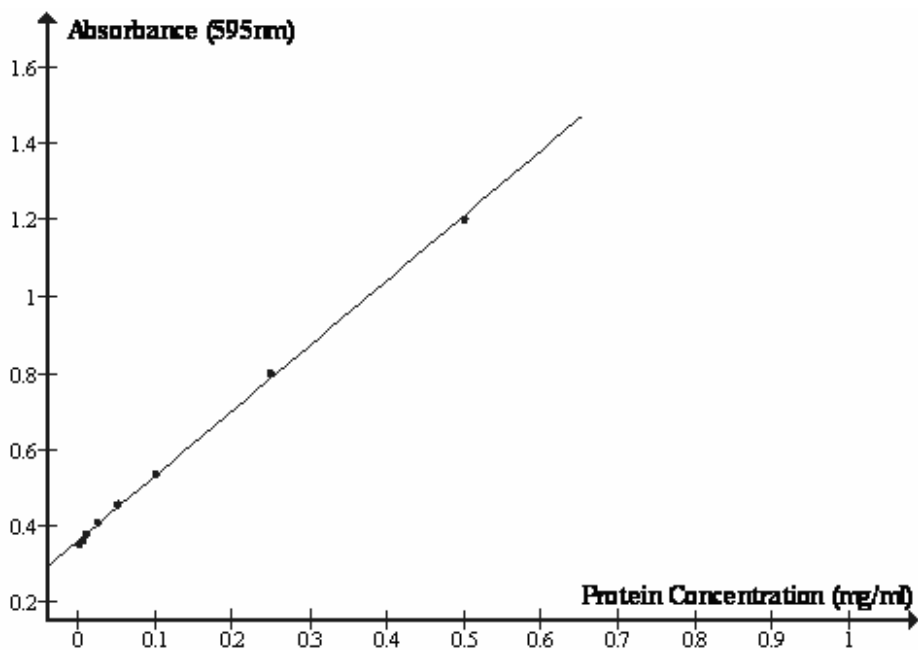
Fig. 4.2:   Graph showing protein standard curve using the Bradford method.

Table 4.4:   Total Protein Concentrations of crude cell lysates

Strain	Absorbance 595nm	Protein Concentration (mg/ml) $f(x) = 1.69682x + 0.3622$
DH2	0.961	19.9287
DH5	0.915	19.1481
DH12	0.681	15.1775
UV5	0.628	14.2782
UV6	0.824	17.6040
AD25	0.995	20.5056
AD27	0.731	16.0259
GJ10	0.541	12.8019

4.3.2 Haloalkane dehalogenase activity

Haloalkane dehalogenase activity is first calculated by quantifying the amount of chlorine liberated in solution during enzymatic cleavage of the carbon chlorine bond of DCA. Chloride ions in solution was measured by firstly determining the absorbance at 460nm of different concentrations of KCl (Table 4.5) using the assay procedure described in Section 4.2.4.1, and generating a standard curve (Fig. 4.3). The generated regression, equation $f(x) = 4.558x2 + 0.908x + 0.042$ was used to determine the amount of chlorine released by each of the isolates at different time intervals in the halide release assay (Tables 4.6 to 4.13). The rate of halide release by each of the isolates over 20 minutes was then obtained from the regression equation generated from the graph constructed of free halide versus time (Fig. 4.4 to 4.11).

The hydrolytic dehalogenase specific activity of each of the isolates was calculated by determining the amount of protein that is required to liberate 1µmol of chlorine per minute under the conditions used. It was found that *A. aquaticus* AD25 had the highest activity (190.351mU/mg protein) toward DCA followed by *A. aquaticus* AD27 (131.271mU/mg protein). Three of the South Africa isolates of *A. aquaticus* (DH2, DH5 and DH12) had higher activities compared to *X. autotrophicus* G10 which had an activity of 66.979mU/mg of protein (Table 4.14). *A. aquaticus* AD25 had approximately 3 fold higher activity as compared to *X. autotrophicus* GJ10 and approximately 2.5 - 3 fold higher activity as compared to the South African isolates of *Ancylobacter*.

Table 4.5: Absorbance at 460nm of different concentrations of KCl

Absorbance (460nm)	Chloride Concentration (mM)
0.572	2
0.547	1.8
0.512	1.6
0.471	1.4
0.435	1.2
0.385	1.0
0.345	0.8
0.285	0.6
0.256	0.5
0.225	0.4
0.159	0.2
0.1	0.1
0.072	0.05
0.05	0.01
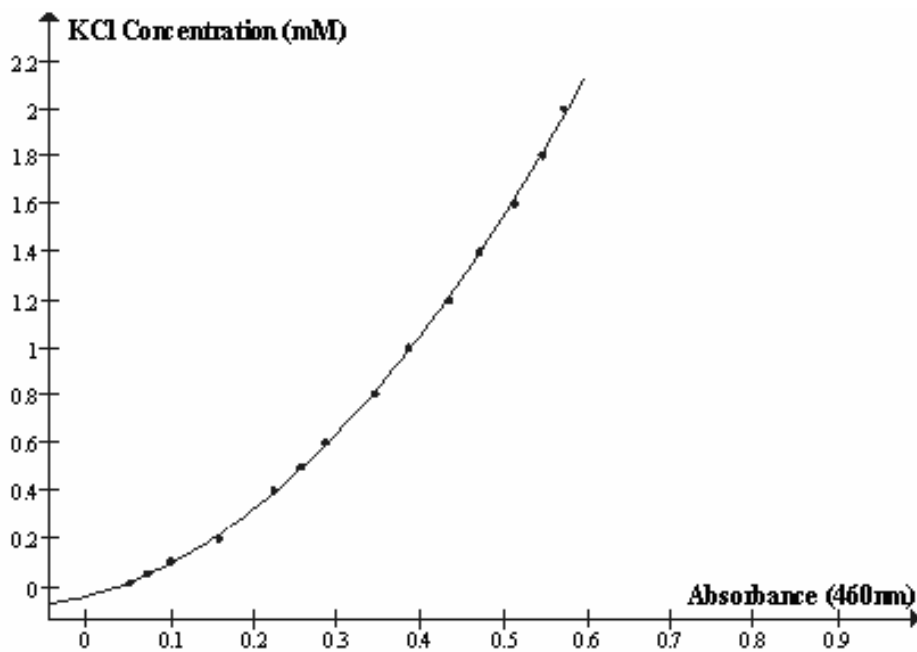
Fig. 4.3: Chloride release standard curve at 460nm.

Strain DH2

Table 4.6: Chloride release by strain DH2 over a 20 min time period using DCA as substrate

Absorbance	Time (min)	Chloride Concentration (mM)
0.195	0	0
0.225	5	0.067
0.235	10	0.112
0.247	15	0.153
0.254	20	0.187
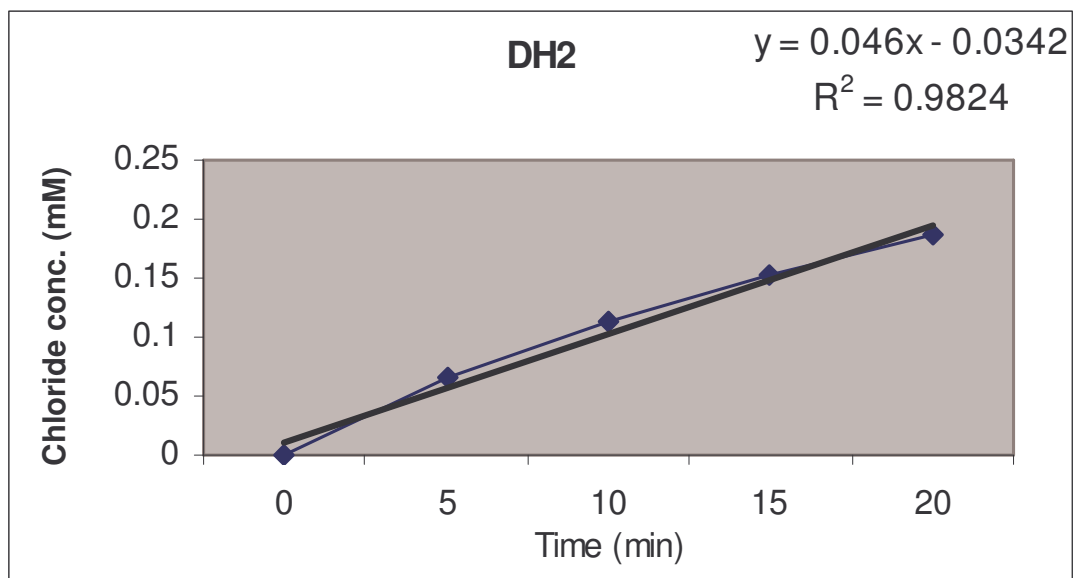
Fig. 4.4: Chloride release by strain DH2 over 20 min time period using DCA as substrate.

Strain DH5

Table 4.7:   Chloride release by strain DH5 over a 20 min time period using DCA as substrate

Absorbance	Time (min)	Chloride Concentration (mM)
0.2	0	0
0.217	5	0.048
0.237	10	0.107
0.252	15	0.154
0.262	20	0.187
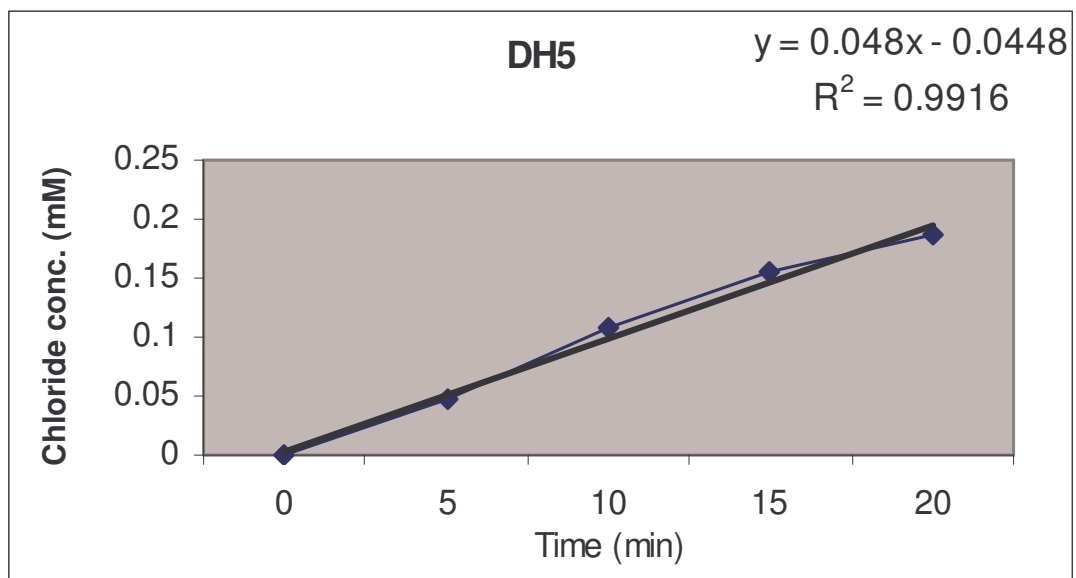
Fig. 4.5:   Chloride release by strain DH5 over 20 min time period using DCA as substrate.

Strain DH12

Table 4.8: Chloride release by strain DH12 over a 20 min time period using DCA as substrate

Absorbance	Time (min)	Chloride Concentration (mM)
0.164	0	0
0.177	5	0.032
0.182	10	0.065
0.19	15	0.097
0.219	20	0.146

DH12

$y = 0.0357x - 0.0391$

$R^2 = 0.9915$
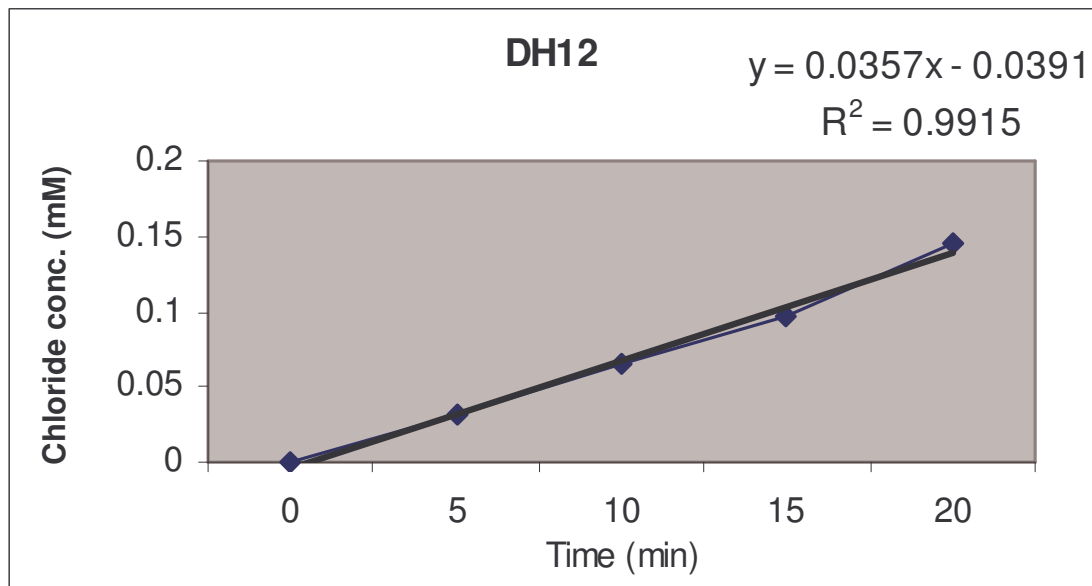
Fig. 4.6: Chloride release by strain DH12 over 20 min time period using DCA as substrate.

Strain UV5

Table 4.9: Chloride release by strain UV5 over a 20 min time period using DCA as substrate

Absorbance	Time (min)	Chloride Concentration (mM)
0.142	0	0
0.167	5	0.04
0.173	10	0.065
0.178	15	0.085
0.189	20	0.114

Fig. 4.7: Chloride release by strain UV5 over 20 min time period using DCA as substrate.

Strain UV6

Table 4.10: Chloride release by strain UV6 over a 20 min time period using DCA as substrate

Absorbance	Time (min)	Chloride Concentration (mM)
0.197	0	0
0.21	5	0.036
0.214	10	0.058
0.22	15	0.085
0.238	20	0.118

Fig. 4.8: Chloride release by strain UV6 over 20 min time period using DCA as substrate.

Strain AD25

Table 4.11: Chloride release by strain AD25 over a 20 min time period using DCA as substrate

Absorbance	Time (min)	Chloride Concentration (mM)
0.213	0	0
0.267	5	0.168
0.304	10	0.297
0.339	15	0.432
0.343	20	0.519

AD25 $y = 0.1302x - 0.1074$ $R^2 = 0.989$

Fig. 4.9: Chloride release by strain AD25 over 20 min time period using DCA as substrate.

Strain AD27

Table 4.12: Chloride release by strain AD27 over a 20 min time period using DCA as

substrate

Absorbance	Time (min)	Chloride Concentration (mM)
0.156	0	0
0.179	5	0.056
0.198	10	0.151
0.202	15	0.213
0.254	20	0.272

AD27 $y = 0.0701x - 0.0719$
$R^2 = 0.9924$
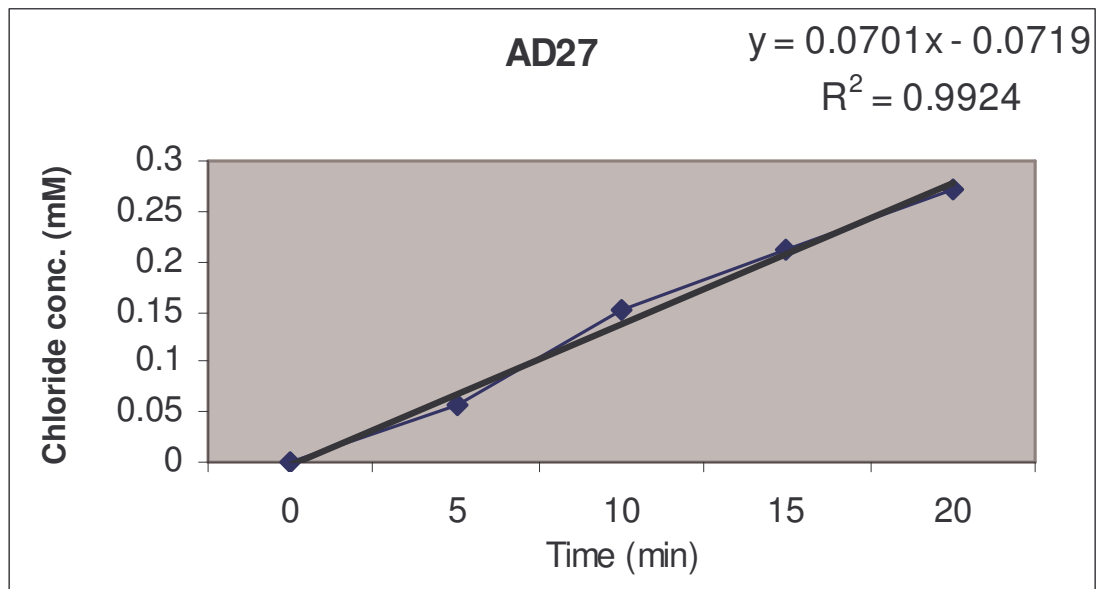
Fig. 4.10: Chloride release by strain AD27 over 20 min time period using DCA as substrate.

Strain GJ10

Table 4.13: Chloride release by strain GJ10 over a 20 min time period using DCA as substrate

Absorbance	Time (min)	Chloride Concentration (mM)
0.126	0	0
0.136	5	0.021
0.149	10	0.05
0.161	15	0.077
0.176	20	0.115
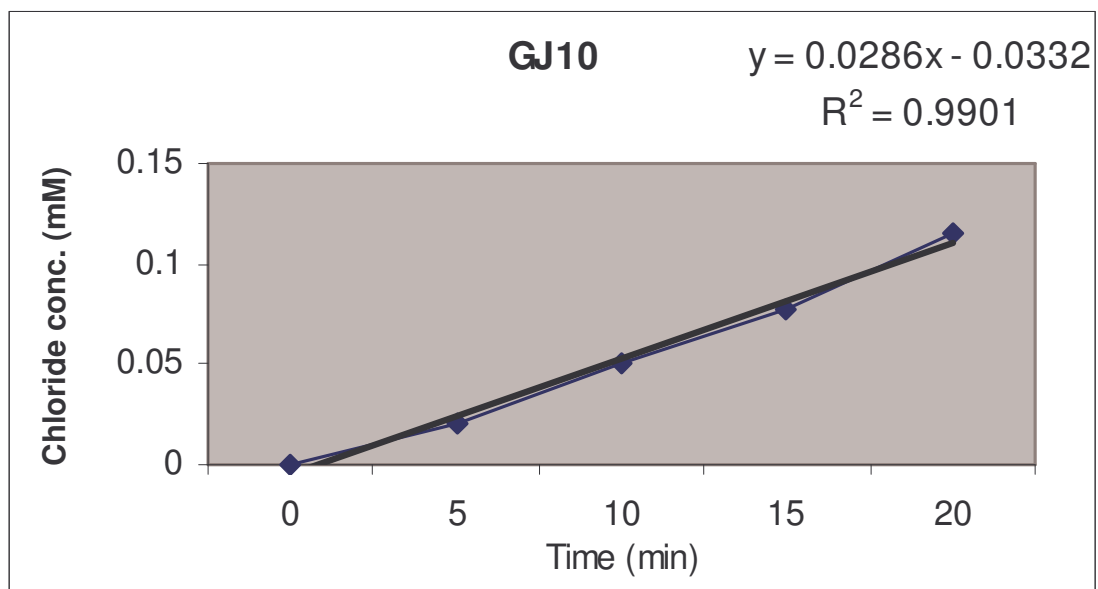
Fig. 4.11: Chloride release by strain GJ10 over 20 min time period using DCA as substrate.

Table 4.14: Hydrolytic dehalogenase specific activity (mU/mg protein) of all isolates using DCA as substrate

Strain	Final Protein Concentration per Reaction (mg/ml)	Reaction Rate (µM/min)	Specific Activity (mU/mg protein)
DH2	0.664	46.0	69.227
DH5	0.638	48.0	75.235
DH12	0.506	37.7	70.553
UV5	0.476	27.3	57.353
UV6	0.587	28.5	48.52
AD25	0.684	130.2	190.351
AD27	0.534	70.1	131.273
GJ10	0.427	28.6	66.979

4.3.3 Haloalcohol dehydrogenase activity

4.3.3.1 2-Chloroethanol

The haloalcohol dehydrogenase specific activity when 2-chloroethanol was used as the substrate in the assay procedure (Table 4.15) was found to be highest in *X. autotrophicus* GJ10 (10.5mU/mg protein) and lowest in *A. aquaticus* AD27 (4.5mU/mg protein). *X. autotrophicus* GJ10 showed an almost two-fold higher activity compared to *A. aquaticus* AD27. Three of the South African isolates (DH2, DH5 and UV5) had comparable activity (7.5, 7.23 and 7.13mU/mg protein, respectively) while isolate DH12 had the highest activity (9.47mU/mg protein).

Table 4.15: Haloalcohol dehydrogenase specific activity (mU/mg protein) of all isolates using 2-chloroethanol as substrate

Strain	Change in absorbance (M/sec)	Protein concentration (mg/ml)	Specific Activity (mU/mg protein)
DH2	0.0055	19.9287	7.5
DH5	0.0051	19.1482	7.23
DH12	0.0052	15.1776	9.47
UV5	0.0037	14.2783	7.13
UV6	0.0030	17.6040	4.77
AD25	0.0062	20.5056	8.20
AD27	0.0024	16.02560	4.5
GJ10	0.0049	12.8020	10.5

4.3.3.2 2-Bromoethanol

The haloalcohol dehydrogenase specific activity when 2-bromoethanol was used as the substrate in the assay procedure (Table 4.16) was found to be highest in *X. autotrophicus* GJ10 (7.4mU/mg protein) and lowest in *A. aquaticus* AD27 (2.05mU/mg protein). *X. autotrophicus* GJ10 showed an almost three-fold higher activity compared to *A. aquaticus* AD27. Among the South African isolates UV6 had the lowest activity (2.33mU/mg protein) while isolate DH12 had the highest activity (6.71mU/mg protein).

Table 4.16: Haloalcohol dehydrogenase specific activity (mU/mg protein) of all isolates using 2-bromoethanol as substrate

Strain	Change in absorbance (M/sec)	Protein concentration (mg/ml)	Specific Activity (mU/mg protein)
DH2	0.0040	19.9287	5.5
DH5	0.0042	19.1482	6.02
DH12	0.0038	15.1776	6.71
UV5	0.0026	14.2783	5.03
UV6	0.0015	17.6040	2.33
AD25	0.0044	20.5056	5.88
AD27	0.0012	16.0260	2.05
GJ10	0.0035	12.8020	7.4

4.3.4 Aldehyde dehydrogenase activity

It was found that the aldehyde dehydrogenase activity was extremely low for all isolates tested (Table 4.17). The highest aldehyde dehydrogenase activity was observed in *X. autotrophicus* GJ10 (36.6mU/mg protein) when 1,2-dichloroethane was used as the carbon source during growth and chloroacetaldehyde was the substrate in the assay procedure. One of the South African isolates of *Ancylobacter* (DH12) had higher activity (33.16mU/mg) compared to the previously characterized isolates AD25 (29.17 mU/mg) and AD27 (14.5mU/mg).

Table 4.17: Aldehyde dehydrogenase specific activity (mU/mg protein) of all isolates using chloroacetaldehyde as substrate

Strain	Change in absorbance (M/sec)	Protein concentration (mg/ml)	Specific Activity (mU/mg protein)
DH2	0.0055	19.9287	26.66
DH5	0.0051	19.1482	25.76
DH12	0.0052	15.1776	33.16
UV5	0.0037	14.2783	24.9
UV6	0.0030	17.6040	16.44
AD25	0.0062	20.5056	29.17
AD27	0.0024	16.0260	14.5
GJ10	0.0049	12.8020	36.6

4.3.5 Haloacetate dehalogenase activity

The haloacetate dehalogenase and haloalkane dehalogenase specific activities are measured in a similar way however in order to measure haloacetate dehalogenase specific activity monochloroacetic acid is used as the substrate. Tables 4.18 to 4.25 show the amounts of chloride released over a 20 minute time period when monochloroacetic acid is used as substrate. The rate of the reaction is derived from the regression equation obtained from the graphs drawn for each isolate showing the amount of free halide released over time (Figs. 4.12 to 4.19). The rate of the free halide liberation (μM/min) is then used to determine the haloacetate dehalogenase specific activity of each of the isolates.

A. aquaticus AD25 had the highest haloacid dehalogenase activity (227.3mU/mg protein) with *X. autotrophicus* GJ10 having an approximately 2.5 fold lower specific activity of 92.9mU/mg protein (Table 4.26). It can be observed that all of the South African isolates of *Ancylobacter* had significantly lower specific activities toward monochloracetate compared to *A. aquaticus* AD25 and AD27. Isolate DH5 had the highest activity (79.310mU/mg protein) among all of the South African isolates.

Strain DH2

Table 4.18   Chloride release by strain DH2 over a 20 min time period using monochloroacetic acid as substrate

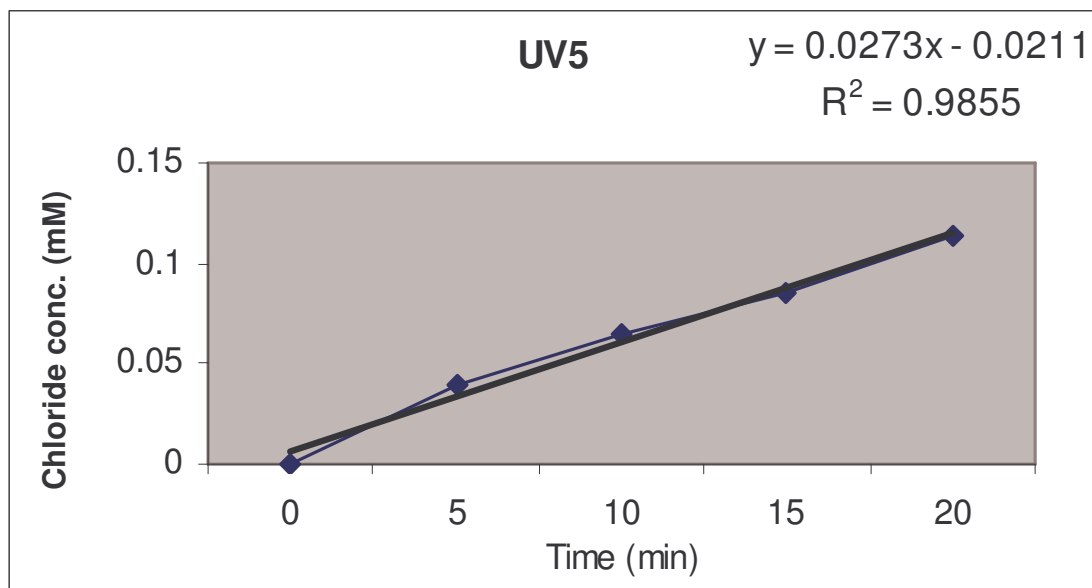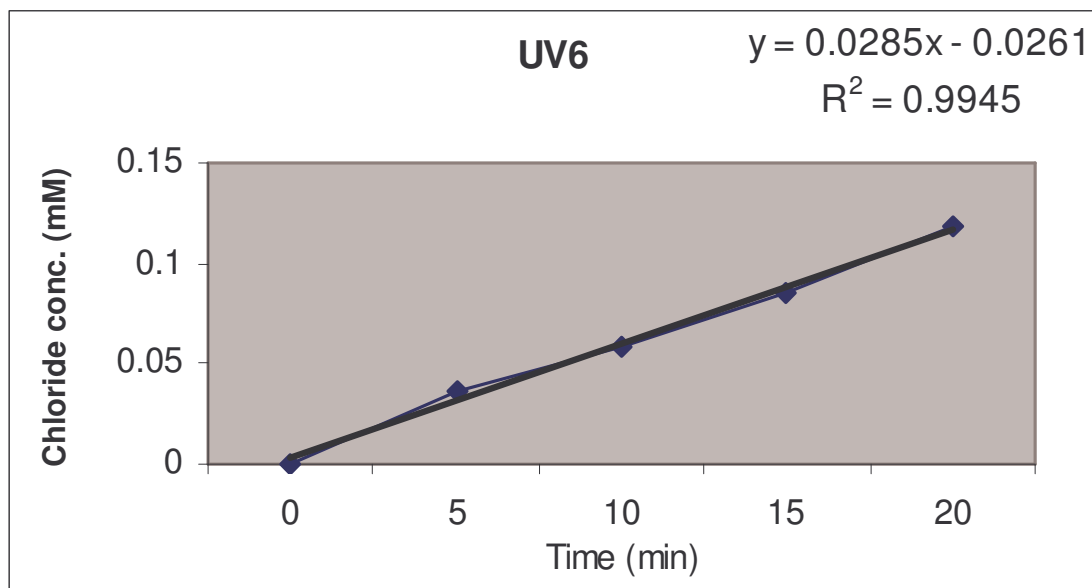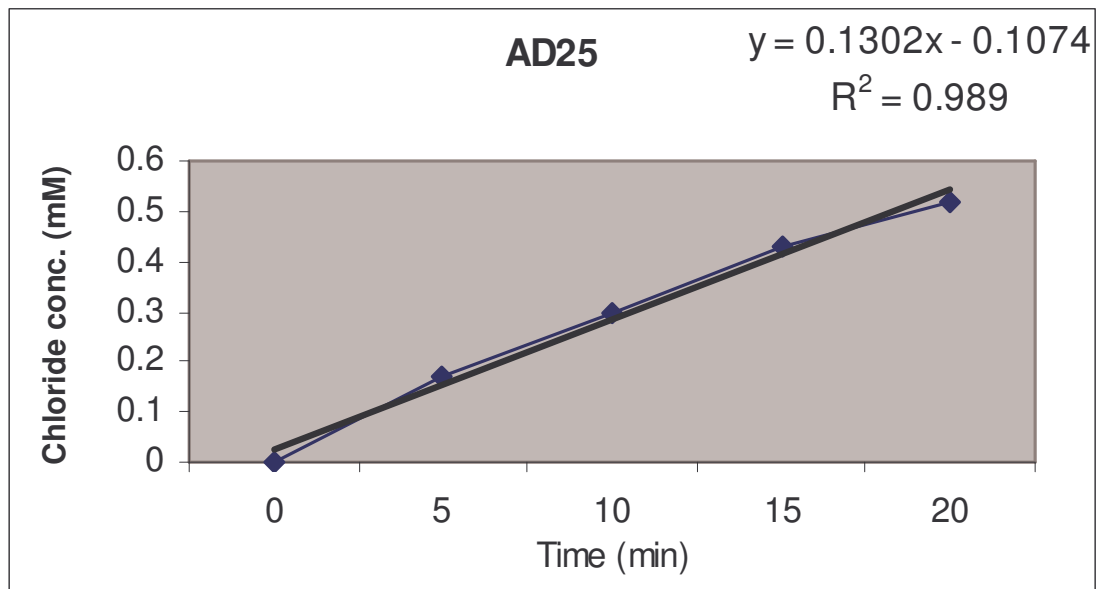| Absorbance	Time (min)	Chloride Concentration (mM)
0.231	0	0
0.24	5	0.028
0.252	10	0.069
0.264	15	0.108
0.279	20	0.157
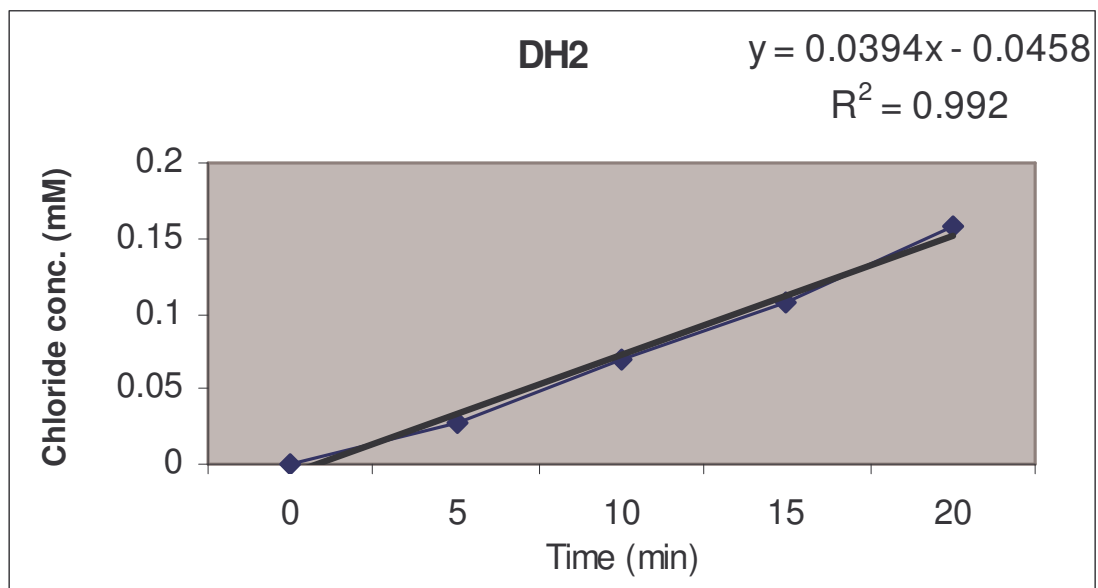
Fig. 4.12:   Chloride release by strain DH2 over 20 min time period using monochloroacetic acid as substrate.

Strain DH5

Table 4.19: Chloride release by strain DH5 over a 20 min time period using monochloroacetic acid as substrate

Absorbance	Time (min)	Chloride Concentration (mM)
0.22	0	0
0.233	5	0.039
0.246	10	0.089
0.257	15	0.137
0.283	20	0.204

DH5

$y = 0.0506x - 0.058$

$R^2 = 0.9911$
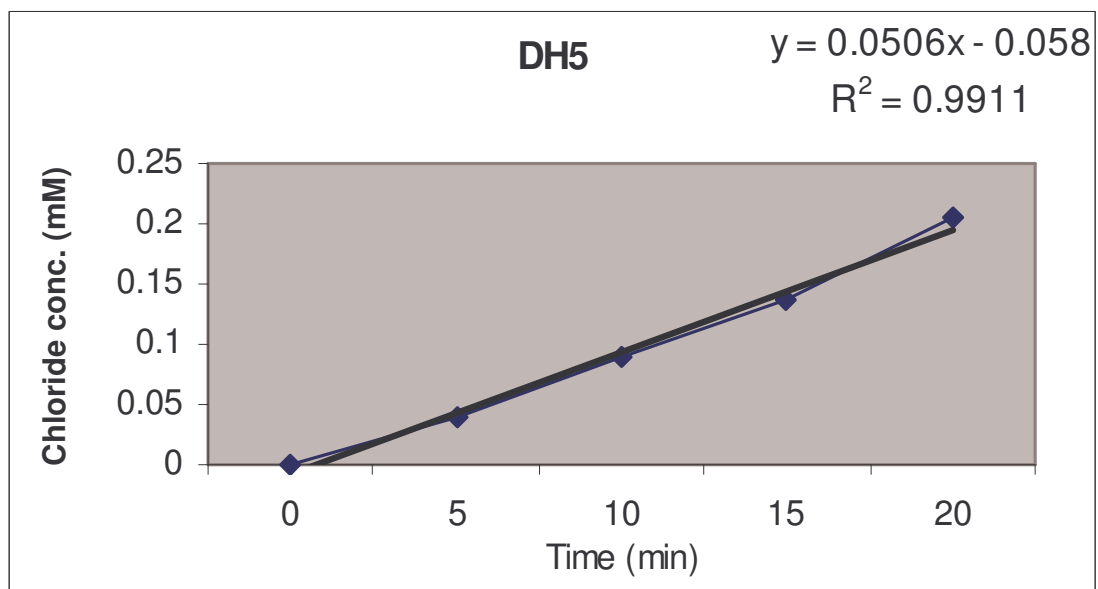
Fig. 4.13: Chloride release by strain DH5 over 20 min time period using monochloroacetic acid as substrate.

Strain DH12

Table 4.20: Chloride release by strain DH12 over a 20 min time period using monochloroacetic acid as substrate

Absorbance	Time (min)	Chloride Concentration (mM)
0.196	0	0
0.2	5	0.021
0.205	10	0.039
0.217	15	0.061
0.228	20	0.092
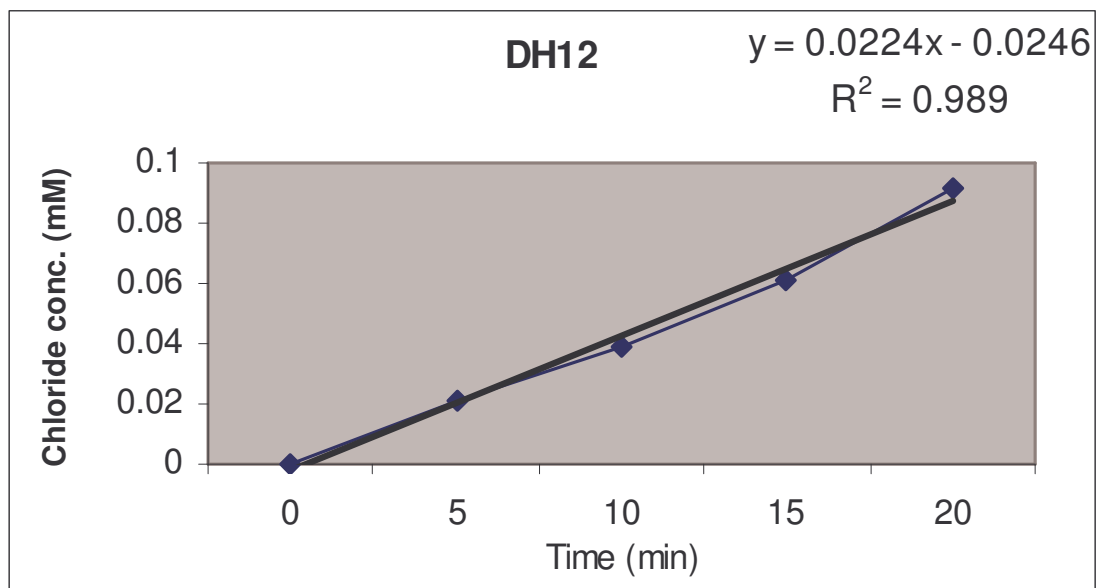
Fig. 4.14: Chloride release by strain DH12 over 20 min time period using monochloroacetic acid as substrate.

Strain UV5

Table 4.21: Chloride release by strain UV5 over a 20 min time period using monochloroacetic acid as substrate

Absorbance	Time (min)	Chloride Concentration (mM)
0.176	0	0
0.181	5	0.018
0.194	10	0.047
0.201	15	0.069
0.214	20	0.103
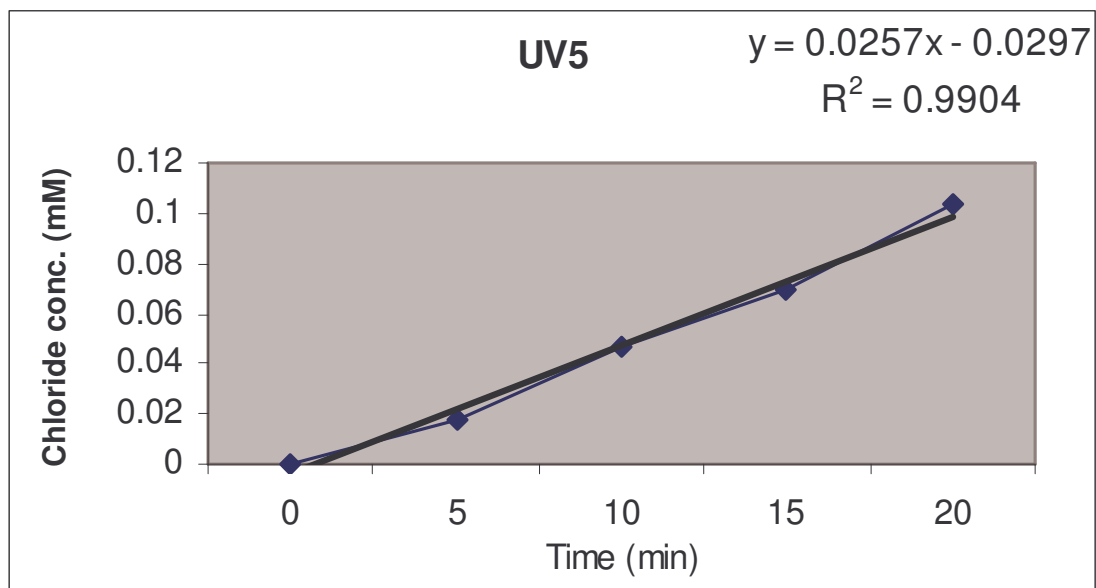
Fig. 4.15: Chloride release by strain UV5 over 20 min time period using monochloroacetic acid as substrate.

Strain UV6

Table 4.22: Chloride release by strain UV6 over a 20 min time period using monochloroacetic acid as substrate

| Absorbance	Time (min)	Chloride Concentration (mM)
0.192	0	0
0.21	5	0.048
0.213	10	0.08
0.233	15	0.118
0.241	20	0.148
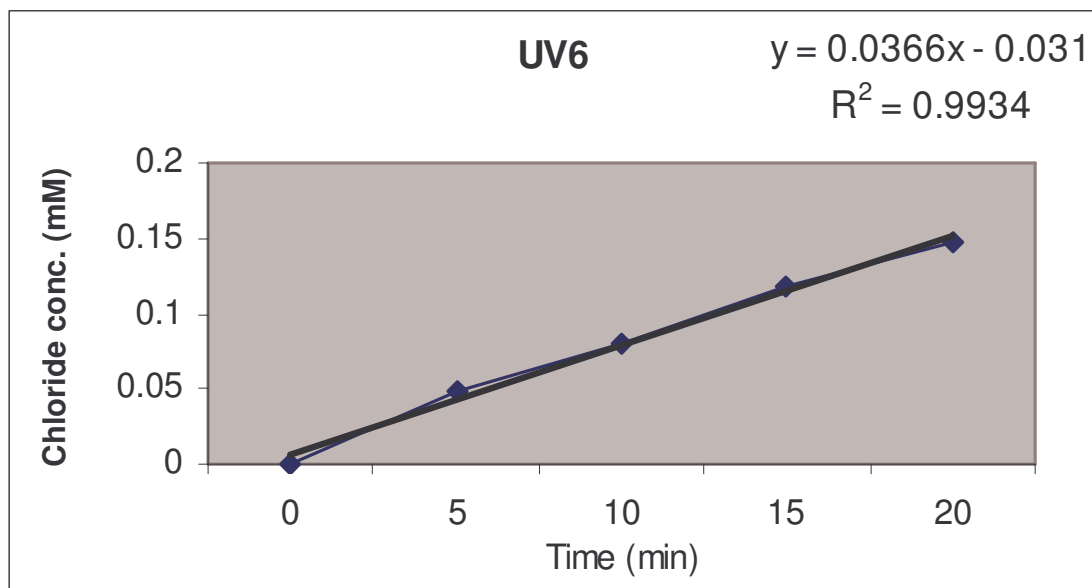
Fig. 4.16: Chloride release by strain UV6 over 20 min time period using monochloroacetic acid as substrate.

Strain AD25

Table 4.23: Chloride release by strain AD25 over a 20 min time period using monochloroacetic acid as substrate

Absorbance	Time (min)	Chloride Concentration (mM)
0.247	0	0
0.31	5	0.219
0.34	10	0.336
0.373	15	0.474
0.412	20	0.65
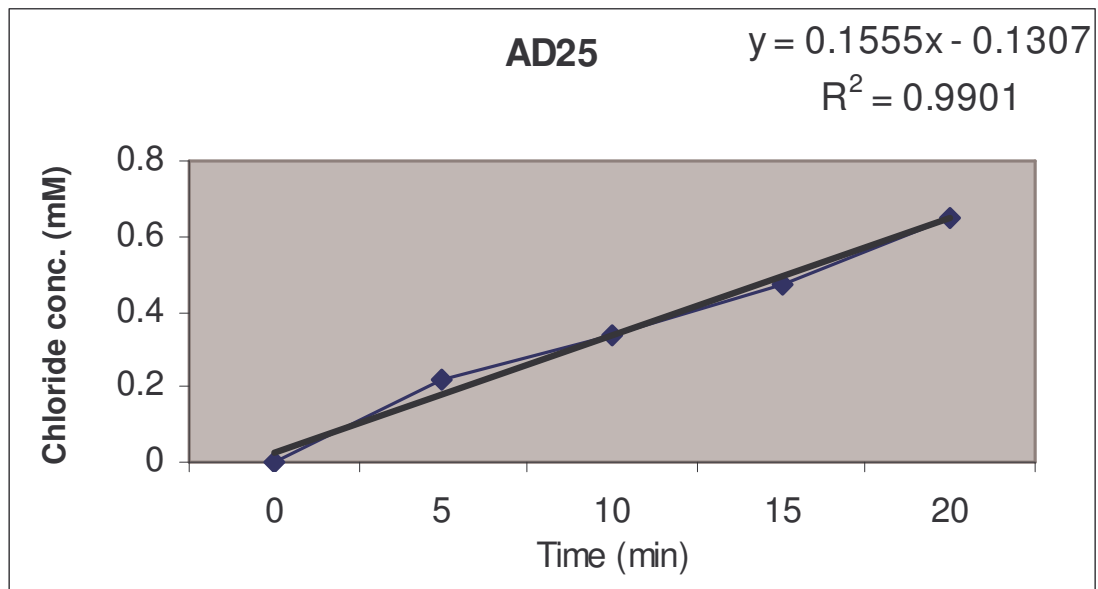
Fig. 4.17: Chloride release by strain AD25 over 20 min time period using monochloroacetic acid as substrate.

Strain AD27

Table 4.24: Chloride release by strain AD27 over a 20 min time period using monochloroacetic acid as substrate

Absorbance	Time (min)	Chloride Concentration (mM)
0.228	0	0
0.259	5	0.098
0.282	10	0.176
0.306	15	0.263
0.33	20	0.355
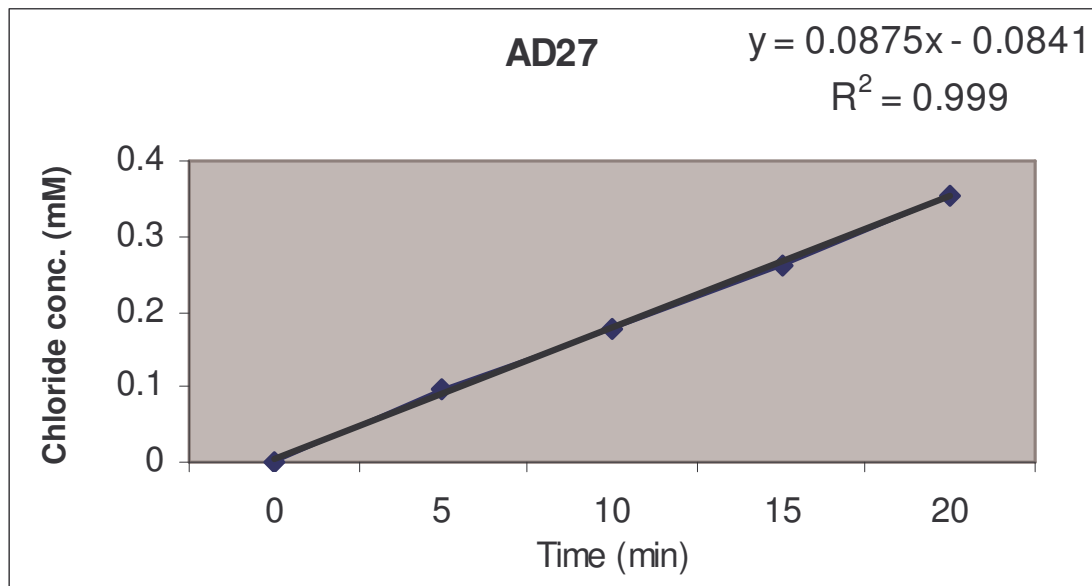
Fig. 4.18: Chloride release by strain AD27 over 20 min time period using monochloroacetic acid as substrate.

Strain GJ10

Table 4.25: Chloride release by strain GJ10 over a 20 min time period using monochloroacetic acid as substrate

Absorbance	Time (min)	Chloride Concentration (mM)
0.178	0	0
0.196	5	0.048
0.209	10	0.084
0.228	15	0.127
0.229	20	0.159
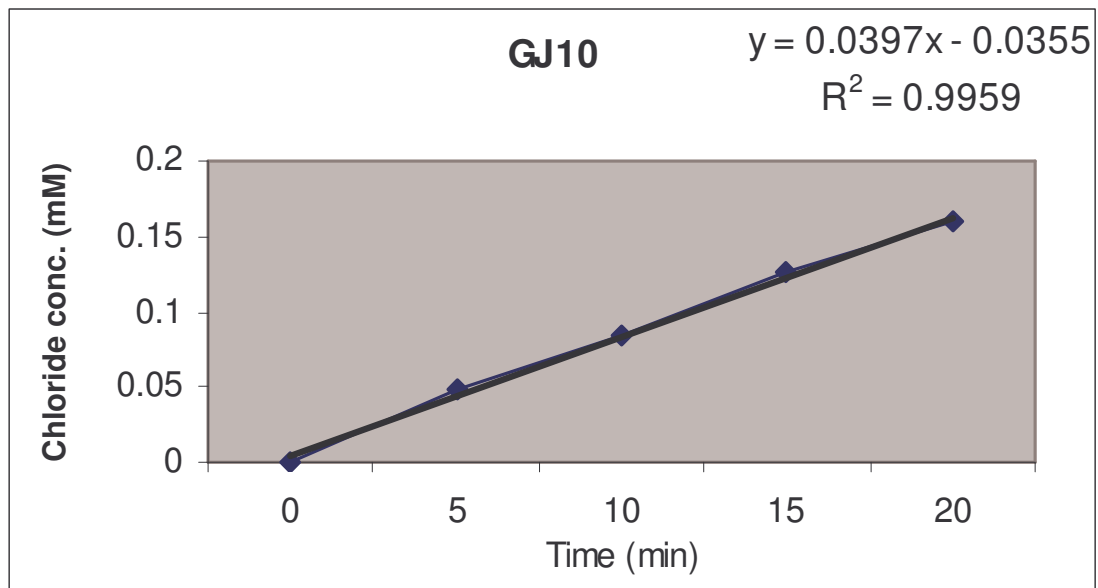
Fig. 4.19: Chloride release by strain GJ10 over 20 min time period using monochloroacetic acid as substrate.

Table 4.26: Haloacetate dehalogenase specific activity (mU/mg protein) of all isolates using monochloroacetic acid as substrate

Strain	Final Protein Concentration per Reaction (mg/ml)	Reaction Rate (μM/min)	Specific Activity (mU/mg protein)
DH2	0.664	39.4	59.337
DH5	0.638	50.6	79.310
DH12	0.506	22.4	44.269
UV5	0.476	25.7	53.992
UV6	0.587	36.6	62.351
AD25	0.684	155.5	227.339
AD27	0.534	87.5	163.858
GJ10	0.427	39.7	92.974

4.3.6 SDS-PAGE analysis
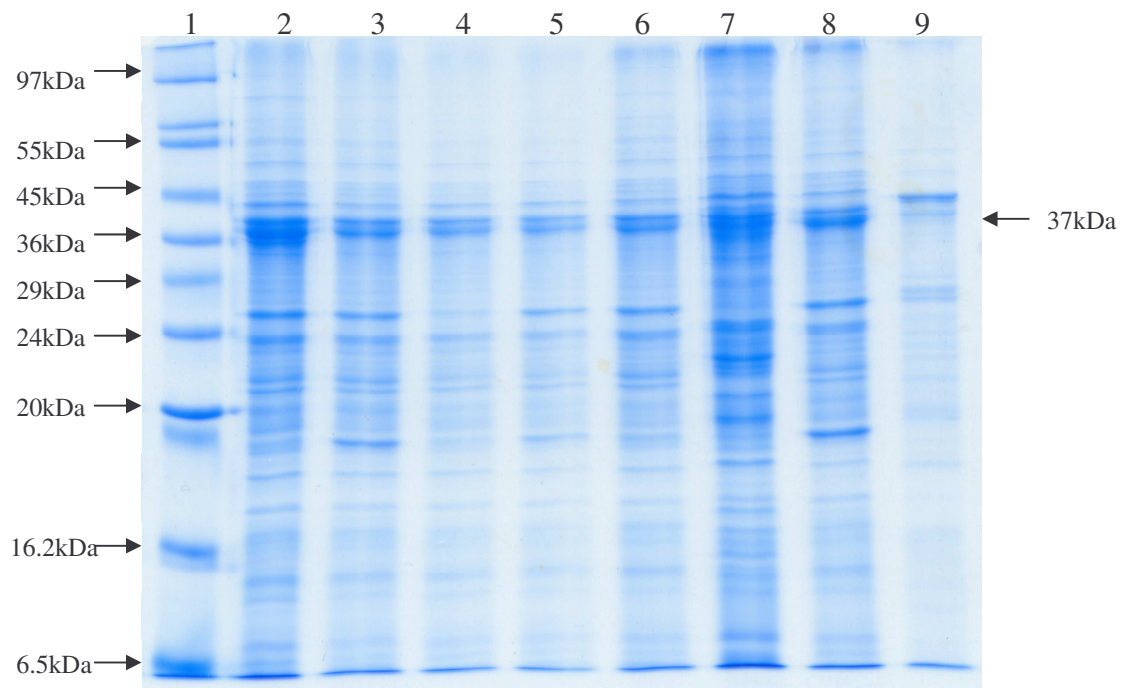
Fig. 4.20: SDS-PAGE analysis of crude cell lysates. Lane 1: Molecular weight marker; lane 2: DH2; lane 3: DH5; lane 4: DH12; lane 5: UV5; lane 6: UV6; lane 7: AD25; lane 8: AD27 and lane 9: GJ10.

SDS-PAGE analysis of all of the isolates was performed in order to determine whether distinct profiling patterns would be observed in the South African isolates compared to the previously characterized isolates *A. aquaticus* AD25 and AD27 (Fig 4.20). *A. aquaticus* AD25 has a unique PAGE profile when compared to the other isolates of *Ancylobacter*. It can be observed that the profile for *X. autotrophicus* GJ10 is different from the isolates of *Ancylobacter* although the intensity of the PAGE profile obtained is low compared to the other isolates which may be due to protein degradation.

Variations in the South African isolates were observed in the 37kDa region for isolate DH2 (lane 2) where an additional band was observed. Isolates DH5, UV5 and *A. aquaticus* AD27 have a more intense 19kDa protein band which was not observed in any of the other isolates. Isolate DH12 (lane 4) lacks a well expressed 25kDa protein which was observed in the other isolates.

4.4 DISCUSSION

The key objective of this chapter was to determine whether the five South African isolates of *A. aquaticus* possessed a similar 1,2-dichloroethane (DCA) degradative route as *X. autotrophicus* GJ10 and two previously characterized isolates of *Ancylobacter aquaticus*, AD25 and AD27. This was achieved using each of the intermediates in the DCA degradative pathway of *X. autotrophicus* GJ10 as substrates in various enzyme assays. The enzyme assays were both a qualitative and quantitative measurement of enzyme activities in crude cell extracts. This allowed for the determination of the exact intermediates in the pathway as well as a comparison to previously characterized DCA degrading microorganisms. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was also carried out in order to compare protein profiles.

It was previously shown that *X. autotrophicus* GJ10 had a specific activity of 232mU/mg total protein when DCA was used as the carbon source during growth and the substrate in the assay procedure (Janssen *et al*., 1985a). This value is significantly higher than the value obtained (66.979mU/mg protein) (Table 4.14). It has previously been shown that pH significantly affected hydrolytic dehalogenase activity. At pH 7.5, activity was shown to be 310mU/mg protein whereas at pH9 activity had decreased to 210mU/mg protein (Janssen *et al*., 1985a). As pH7.5 was used it can be assumed that the lower activity was not due to the pH but possible protein degradation.

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Initial studies that analysed the dehalogenase of isolates of *A. aquaticus* had shown that isolate AD25 had a specific activity of 4293mU/mg total protein which is 15 to 20 times higher than that of *X. autotrophicus* GJ10 (232mU/mg protein) when 1,2-dichloroethane was used as the carbon source during growth and the substrate in the assay procedure while isolate AD27 had a specific activity of 936mU/mg protein (van den Wijngaard *et al.*, 1992). The highest activity for the South African isolates was observed in isolate DH5 (75.235mU/mg protein) which is approximately 57-fold lower than *A. aquaticus* AD25 and 12 fold lower than *A. aquaticus* AD27 (Table 4.14).

The specific activity of the haloalcohol dehydrogenases from *X. autotrophicus* GJ10 and *A. aquaticus* AD25 towards 2-chloroethanol were found to be much lower than previously observed (Janssen *et al.*, 1985a; van den Wijngaard *et al.*, 1992). In this study (Table 4.15), *X. autrophicus* GJ10 had a specific activity of 10.5mU/mg protein while in previous studies the specific activity was shown to be 518mU/mg protein (Janssen *et al.*, 1985a) and 480mU/mg protein (van den Wijngaard *et al.*, 1992) when DCA was used as the carbon source during growth and 2-chloroethanol was the substrate in the assay procedure. *A. aquaticus* AD25 had a specific activity of 260mU/mg protein (van den Wijngaard *et al.*, 1992) while in this study (Table 4.15) the specific activity was 8.2mU/mg protein. In this study one of the South African isolates (DH12) had higher specific activity (9.47mU/mg protein) compared to the previously characterized isolates of *A. aquaticus* AD25 (8.2 mU/mg protein) and AD27 (4.5mU/mg protein).

When bacterial isolates were grown with DCA as the carbon source but 2-bromoethanol was used as the substrate in the haloalcohol dehydrogenase assay (Table 4.16), it was found that *X. autotrophicus* GJ10 had the highest activity (7.4mU/mg protein) whereas *A. aquaticus* AD27 had the lowest activity (2.05mU/mg protein). Two of the South African isolates of *A. aquaticus* (DH5 and DH12) had higher activities than the previously characterized isolates of *Ancylobacter*, 6.02mU/mg protein and 6.71mU/mg protein, respectively. These values are much lower than previously observed values for *A. aquaticus* AD25 which had previously been shown to have an activity of 263mU/mg protein (van den Wijngaard *et al*., 1992). All bacterial isolates seem to posses a higher activity toward 2-chloroethanol compared to 2-bromoethanol (Tables 4.15 and 4.16).

Chloroethanol and chloroacetaldehyde are two of the most toxic intermediates in the degradative pathway and have to be rapidly converted or removed from the cell. It has been observed that 2-chloroethanol can be excreted from the cell while toxic aldehydes generally accumulate with the cell (Janssen *et al*., 1985a). Both the alcohol dehydrogenase and aldehyde dehydrogenase are therefore key regulatory enzymes required to prevent the accumulation of toxic intermediates. Although the South African isolates have lower chloroacetaldehyde dehydrogenase activities compared to the previously characterized isolates of *A. aquaticus* AD25 and AD27, isolate DH12 (Table 4.17) has a higher compared to both of these isolates. Previous studies have shown specific activity of *X. autotrophicus* GJ10 to be 109mU/mg protein (Janssen *et al*., 1985a) while in this study it was 36.6mU/mg protein. The isolates of *A. aquaticus* AD25 had a specific activity of 1872mU/mg total protein while *A. aquaticus* AD20 (which has similar

activity to AD27) had a specific activity of 109 mU/mg protein (van den Wijngaard *et al.*, 1992). The lower activity towards chloroethanol and chloroacetaldehyde may be due to the inhibitory effects of these highly reactive intermediates (Table 4.17).

Lastly, the specific activity of the haloacid dehalogenases was found to be extremely low for all the isolates tested (Table 4.18). In previous studies the specific activity of the haloacid dehalogenase from *X. autotroph*icus GJ10 was shown to be 416mU/mg protein (Janssen *et al.*, 1985a) while the specific activity of the haloacid dehalogenase from *A. aquaticus* AD25 was approximately 2.5 fold higher at 1035mU/mg protein (van den Wijngaard *et al.*, 1992). In this study, highest haloacid dehalogenase activity was observed in *A. aquaticus* AD25 (227.3mU/mg protein) with *X. autotrophicus* GJ10 having an approximately 2.5 fold lower specific activity of 92.9mU/mg protein. All of the South African isolates of *Ancylobacter* had much lower specific activities toward monochloracetate (Table 4.18).

It has previously been shown that *A. aquaticus* AD25 usually over-expresses the hydrolytic dehalogenase (van den Wijngaard *et al.*, 1992) which could be observed in a slightly more intense 37kDa band in Fig. 4.20. Based on the hydrolytic dehalogenase enzyme assay (Table 4.14), it is also observed that *A. aquaticus* AD25 has a much higher activity compared to the other isolates of *Ancylobacter* and this may be due to enhanced expression of enzyme. The determination of whether other proteins involved in the degradation pathway are expressed at high levels is not possible as none of the other enzymes besides the hydrolytic dehalogenase have been previously identified in isolates

of *A. aquaticus*. Differences observed in the protein profiles of the different isolates of *Ancylobacter* indicate that these isolates express proteins differently and may thus exhibit different degradation potentials.

Lower activities toward some of the compounds may be attributed to poor enzyme stability over extended periods of time or sub-optimal assay conditions. By performing assays together with known controls, specific activities can be compared even with reduced activities. The lower values for the specific activities of the different enzymes obtained in this study may be due to protein degradation in crude cell lysates during overnight storage at 4°C. Loss of protein expression may also be attributed to repeated sub-culturing in undefined media prior to growth in media containing DCA as a sole carbon and energy source. This study has been adequately able to demonstrate that the South African isolates of *Ancylobacter* have a similar degradative route to *X. autotrophicus* GJ10 as well as two previously characterized isolates of *Ancylobacter*.

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CHAPTER FIVE

PFGE AND RAPD PROFILING AS A MEANS OF DISTINGUISHING *Ancylobacter aquaticus* ISOLATES CAPABLE OF 1,2-DICHLOROETHANE (DCA) DEGRADATION

5.1   INTRODUCTION

The widespread and continued use of synthetic haloalkanes in industry and agriculture, both locally and internationally, has resulted in the evolution of several bacterial enzymes that possess the ability to degrade some of these compounds (Janssen *et al*., 2005). The evolution of bacterial genes is generally a slow process and results mainly from prolonged exposure to these compounds (van der Meer *et al*., 1992). However, in some cases the possession of these enzymes may not necessarily result from exposure to xenobiotic compounds. In these cases the enzymes may be involved in certain vital metabolic processes but somehow recognize synthetic compounds as substrates and thus possess the ability to degrade these compounds either partially or completely (Copley, 2000).

Certain bacteria may be restricted to ecological niches and may neither survive nor be present in large enough numbers outside of their evolutionary niches whereas others may be present in varied niches, due to the ability to utilize a wide variety of substrates. It can be assumed that once a bacterium possesses or has evolved a catabolic system to utilize a

particular substrate as carbon and energy source it would now possess the ability to survive in a biological environment where the substrate is present, provided that there are no other growth limiting factors present. Once dissemination by horizontal gene transfer has occurred, no changes may occur in the genes coding for the ability to utilize the substrate but genotypic changes may occur due to natural genetic events rendering descendants distinguishable from the original bacterium at a genotypic level (van der Meer *et al.*, 1992).

New environmental isolates of known synthetic haloalkane degrading bacteria from different geographic regions may be genetically identical or closely related (Janssen *et al.*, 2005). In many cases, closely related isolates may have evolved from a common ancestor and are thus clonally related. Although these bacteria are members of the same species that share virulence factors, biochemical traits and genomic characteristics, there is sufficient diversity at the species level that organisms isolated from different sources at different times and in different geographical regions may be differentiated or even classified into subtypes or strains (Olive and Bean, 1999).

Phylogenetic analysis of different environmental isolates may provide enough information in order to determine how closely or distantly related a set of isolates may be. This process may be able to identify an ancestral isolate from a specific geographical region from which all other isolates may have diverged or acquired genes due horizontal gene transfer. A higher level of genotypic divergence from an ancestral strain may be due to prolonged exposure to a number of xenobiotic compounds and this may result in

the development of novel catabolic pathways involved in the degradation of halogenated hydrocarbons (Springael and Top, 2004). Identifying and typing new isolates may thus prove to be a vital tool in the characterization of novel catabolic genes and pathways (Rison and Thornton, 2002).

The choice of method used for the typing of environmental bacterial isolates is dependant on a number of factors. Analysis of the DNA sequence of catabolic genes from different isolates is one way of determining genetic relationship between the individual isolates at gene level. Over time genetic changes at the gene level may have occurred to alter the microorganism's ability to utilize a particular substrate. Analysis of the entire genome may provide more information as a larger subset of genes can be compared using a variety of molecular based approaches (Janssen *et al*., 2005).

There are a number of different molecular typing methods currently available. Each method has its own relative advantages and disadvantages therefore the method of choice should meet several criteria to be useful. Firstly, the method of choice should ensure that all organisms within a species are typeable. Serological methods such as those based on the reaction with a specific antibody or the presence of a bacteriophage receptor may not always be feasible as these characteristics may not be present in all members. Secondly the method should have the ability to clearly differentiate strains from different geographic regions from the source organism and at the same time demonstrate the relatedness of all isolates. Thirdly the method should be reproducible. This refers to the ability of the technique to yield the same result after being performed several times.

Phenotypic characteristics relying on the expression of various genes may produce variable results and may not produce reproducible results especially for the construction of strain databases. PCR-based methods such as RAPD fingerprinting may also produce variable results should amplification conditions not be carefully controlled and may have to be repeated several times to ensure reproducibility (Williams *et al*., 1990). At present molecular methods based on the genotype or DNA sequence of individual isolates are generally preferred to phenotype-based methods due to improved reproducibility of the former method under carefully controlled conditions (Olive and Bean, 1999).

Electrophoretic molecular techniques involve the separation of DNA fragments of varying molecular length across an electric field. The resulting pattern of bands obtained for each isolate is then analysed either manually for simple patterns or using various computer programs for more complex patterns. Two of the more common methods of strain typing include randomly amplified polymorphic DNA (RAPD) and pulsed field gel electrophoresis (PFGE) (Olive and Bean, 1999).

Schwartz and Cantor, (1984) first developed the PFGE technique which is widely used for bacterial typing and is generally referred to as the "gold standard" of molecular typing methods. Agarose plugs are prepared by first growing individual isolates on solid media or in broth and combining the cells with molten agarose which is poured into special molds. Once solid, the plugs containing embedded bacteria are then treated with enzymes and detergents to lyse the cells. Liberated DNA within the plugs is then digested with restriction endonucleases that cleave infrequently. Plugs are then inserted

into wells in an agarose gel and subjected to electrophoresis in a tank where the direction of the current is changed at regular intervals allowing for the separation of large molecular length DNA fragments. Following staining with fluorescent dyes the electrophoretic patterns (fingerprint) of each of the isolates are recorded and compared.

The RAPD assay also called arbitrary primed PCR (Welsh and McClelland, 1990; Williams *et al*., 1990) is a simple and fast technique for genetic analysis. This PCR based technique uses short primers with arbitrary sequences, 9 to 10 bases in length, to bind to genomic DNA at low annealing temperatures to amplify specific regions within the genome. The single primer used in each reaction needs to bind to DNA within a few kilobases in the correct orientation in order to generate a PCR product. The distance between priming sites varies depending on the relationship between strains. Following electrophoretic separation on agarose gels and staining, the pattern generated is then recorded and compared.

In this chapter PFGE and RAPD analysis have been used in order to phylogenetically compare five South African isolates of *Ancylobacter aquaticus* with two previously characterized isolates. Standard protocols have been used for both techniques in order to compare the efficiency of each technique to discriminate between strains. In order to analyse the data obtained, the banding patterns generated for each of the isolates have been compared. The percentage similarity between two strains were then estimated using various algorithms and dendograms were constructed from the percentage similarity values.

5.2 MATERIALS AND METHODS

5.2.1 Bacterial isolates

Table 5.1 lists the bacterial isolates capable of haloalkane degradation used in this study. *Ancylobacter aquaticus* AD25 and AD27, isolated from the Eems channel near Delfzijl, The Netherlands (van den Wijngaard *et al*., 1992) and *Xanthobacter autotrophicus* GJ10 isolated from a mixture of activated sludge and soil from chemically polluted sites in Groningen, The Netherlands (Janssen *et al*., 1984) were used as reference strains.

Table 5.1:   Bacterial isolates used in this study

Bacterial isolate	Source
Ancylobacter aquaticus DH2	This study
Ancylobacter aquaticus DH5	This study
Ancylobacter aquaticus DH12	This study
Ancylobacter aquaticus UV5	This study
Ancylobacter aquaticus UV6	This study
Ancylobacter aquaticus AD25	Dick B. Janssen*
Ancylobacter aquaticus AD27	Dick B. Janssen*
Xanthobacter autotrophicus GJ10	Dick B. Janssen*

Dick B. Janssen* - Biochemical Laboratory, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

5.2.2 PFGE

5.2.2.1   Growth of bacterial cultures

Bacterial cultures were all grown at 30ºC in 10ml LB broth medium (10g tryptone, 5g yeast extract and 5g NaCl per litre) with shaking for 16 hours.

5.2.2.2   Preparation of DNA in agarose plugs

Bacterial cells were harvested by centrifugation (8000 x g/10min). Pelleted cells were then resuspended with an equal volume of wash buffer (10mM Tris.Cl, 1mM EDTA, 10mM NaCl) and centrifuged (8000 x g/10min). Cells were then re-suspended in 1/5 original volume of wash buffer. An equal volume of 2% LMP agarose (Sigma) in 0.5 x TBE cooled to approximately 42-50ºC was then added to the cell suspension and mixed by vortexing. Suspensions were then immediately poured into sealed rectangular slots (4 x 3 x 12mm) of an agarose plug mould. Moulds containing plugs were then cooled at 4ºC to ensure quick and even solidification (Poh and Lau, 1993).

Plugs were then transferred to McCartney bottles containing 5ml lysis buffer (6mM Tris-Cl, 100mM EDTA, 1M NaCl, 0.5%Triton-X100, 0.2% sodium deoxycholate, 0.5% sodium lauroyl sarcosine, 10mg lysozyme and 5mg/ml RNase). Overnight incubation at 37ºC ensured efficient lysis of cells embedded within plugs. Plugs were then transferred to fresh bottles containing 5ml proteolysis buffer (0.5M EDTA [pH 9.0], 1% sodium lauroyl sarcosine, 50µg/ml proteinase K) and incubated overnight at 55ºC. The proteinase K was responsible for degradation of cellular proteins while the detergent aided in cell lysis. The plugs were then transferred to fresh bottles and washed three

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times with 5ml TE buffer (10mM Tris-Cl [pH7.5], 0.1M EDTA). Plugs were stored at 4ºC in TE buffer until required (Poh and Lau, 1993).

5.2.2.3   Restriction of DNA in agarose plugs

In order to determine whether the isolates showed any polymorphisms, total DNA embedded in LMP agarose (Sigma) plugs were cleaved with different restriction endonucleases and analysed. Prior to digestion agarose plugs were first equilibrated overnight in 1ml sterile deionised water at 4ºC. Plugs were then also equilibrated in 1 x of the appropriate restriction endonuclease buffer for 5 hours. Genomic DNA within the plugs was then restricted for 16 hours at 37ºC. Restriction reactions typically contained in a final volume of 100µl: 1 x restriction buffer, 50U of restriction endonuclease, agarose plug (2 x 4 x 4mm) brought up to 100µl with sterile deionised water. Plugs were restricted with the following restriction enzymes (Roche): *Xba*I, *Pst*I, *Cla*I, *Hin*dIII, *Bam*HI and *Xho*I.

5.2.2.4   Electrophoresis of DNA in agarose plugs

Following restriction, agarose plugs were loaded into wells of a 1% PFGE grade agarose gel (Biorad) in 0.5 X TBE electrophoresis buffer. Wells were first flooded with 0.5 X TBE buffer to ensure efficient loading. After loading wells were sealed with 1% agarose prepared with 0.5 X TBE electrophoresis buffer. The electrophoresis tank was filled to the required level with 0.5 X TBE electrophoresis buffer and pre-chilled to 15ºC prior to placing the gel within the tank. Plugs were subjected to PFGE in a ChefMapper (BIORAD) system at 6V/cm for 15 hours with the initial and final switch times of 1-12s,

respectively, at an angle of 120º and a linear ramp. The temperature was maintained at 15ºC throughout the period of the run with a chilling unit (BIORAD). Following electrophoresis, gels were stained in ethidium bromide and documented using the ChemiGenius Gel Imaging System (Syngene).

5.2.3   RAPD

5.2.3.1   Isolation of bacterial DNA

Bacterial DNA was isolated and quantified as described in Section 2.2.7.2.

5.2.3.2   PCR conditions and electrophoresis

The six 10-mer RAPD primers used in this study (Table 5.2), based on sequences from the University of British Columbia RAPD Primer Synthesis project were synthesized by Inqaba Biotech, South Africa.

Table 5.2:   Sequences of 10-mer primers used in RAPD reactions

Primer	Mol% G + C	Sequence (5' - 3')
UBC237	70	CGACCAGAGC
UBC208	80	ACGGCCGACC
UBC228	80	GCTGGGCCGA
UBC275	80	CCGGGCAAGC
UBC245	80	CGCGTGCCAG
UBC210	70	GCACCGAGAG

Stock solutions of primers were prepared by the addition of the appropriate amount of sterile de-ionised water to create a 1M stock while working stocks were at 100mM. All primer stocks were stored at -20°C until required. The PCR mixtures (50µL) contained 10ng DNA, 100pmol of primer, 100µM of each of the deoxynucleoside triphosphates (dNTPs), 1 x Super-therm Taq polymerase buffer and 0.5U Super-therm Taq polymerase (Southern Cross Biotech) finally brought to volume with sterile de-ionised water. PCR was performed using the PE Applied Biosystems GeneAmp PCR System 9700 (Perkin Elmer) programmed to perform an initial denaturation at 94°C for 5min and 30 cycles consisting of 94°C for 2min, 36°C for 1min and 72°C for 2min followed by a final extension step of 72°C for 10min. PCR products were fractionated on a 2% agarose gel, stained with ethidium bromide and documented using the Chemi-Genius Bio Imaging System (Syngene) (Welsh and McClelland, 1990). Each RAPD analysis was performed three times in order to ensure reproducibility. Primers producing reproducible results are indicated in Table 5.2.

5.2.4   Analysis of PFGE and RAPD gels

Images of both PFGE ad RAPD gels were analysed using the GelCompar version 4.1 software (Applied Maths) as described by the manufacturer's instructions. The Pearson product moment correlation coefficient (r) and the unweighted pair group method of arithmetic averages (UPGMA) algorithm within GelCompar was then used to construct dendograms from the similarity matrices (Jackson *et al*., 1989).

5.3 RESULTS

5.3.1 RAPD analysis

Genomic DNA isolated from all haloalkane degrading bacteria was successfully amplified using the RAPD primers. The RAPD profile generated by RAPD primer UBC237 (Fig. 5.1) indicated that isolates DH5 and DH12 are 100% identical but are only 84% similar using primer UBC208 (Fig. 5.2), 83% similar using UBC228 (Fig. 5.3), 70% similar using primer UBC275 (Fig. 5.4), 90% similar using primer UBC245 (Fig. 5.5) and 80% similar using primer UBC210 (Fig. 5.6).

The profile generated by primer UBC208 (Fig. 5.2) indicates that isolates UV6 and AD27 are identical while the profiles generated for primers UBC237 (Fig. 5.1), UBC228 (Fig. 5.3), UBC275 (Fig. 5.4), UBC245 (Fig. 5.5) and UBC210 (Fig. 5.6) indicates an 86%, 90%, 85%, 72% and 93% similarity, respectively.
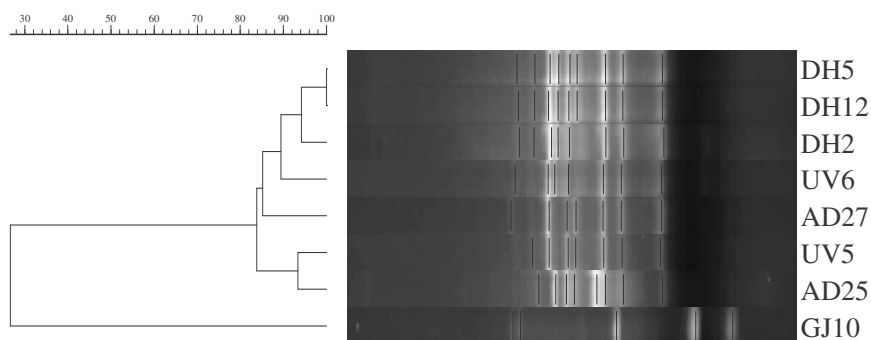
Fig. 5.1:   Dendogram showing the percentage of similarity between isolates based on computer comparisons of RAPD patterns produced using RAPD primer UBC237. *X. autotrophicus* GJ10 was used as the outgroup.
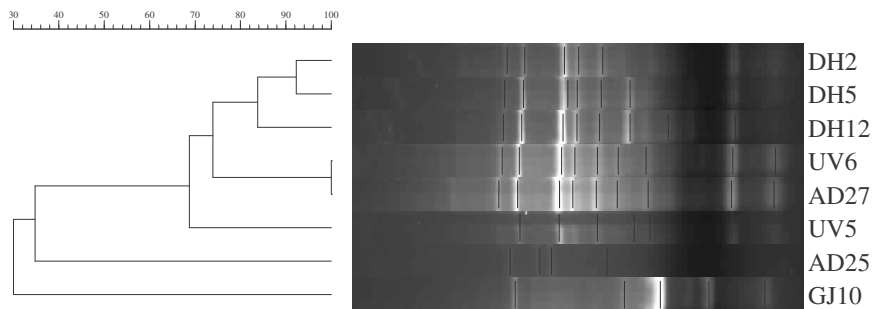
Fig. 5.2: Dendogram showing the percentage of similarity between isolates based on computer comparisons of RAPD patterns produced using RAPD primer UBC208. *X. autotrophicus* GJ10 was used as the outgroup.

The profiles of isolates DH5 and AD27 indicate 100% identity for primer UBC228 (Fig. 5.3). These isolates show 85% similarity for primer UBC237 (Fig.5.1), 74% similarity for primer UBC208 (Fig.5.2), 80% identity using primer UBC275 (Fig. 5.4), 72% identity using primer UBC245 (Fig. 5.5) and 85% similarity using primer UBC210 (Fig. 5.6).
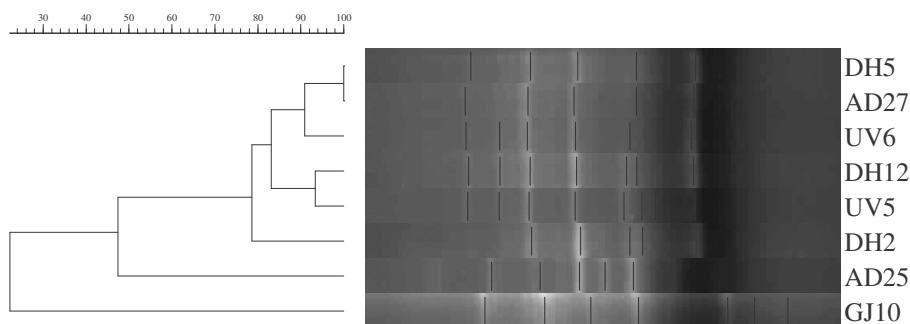
Fig. 5.3: Dendogram showing the percentage of similarity between isolates based on computer comparisons of RAPD patterns produced using RAPD primer UBC228. *X. autotrophicus* GJ10 was used as the outgroup.

The RAPD primer UBC245 (Fig. 5.5) produced profiles of 100% identity for isolates DH2 and DH5 while at the same time indicating that isolates UV5 and UV6 are also 100% identical. Isolates DH2 and DH5 show 94% similarity using primer UBC237 (Fig. 5.1), 92% similarity using primer UBC208 (Fig. 5.2), 79% homology using primer UBC228 (Fig. 5.3), 82% homology using primer UBC275 (Fig. 5.4) and 92% homology using primer UBC210 (Fig. 5.6). Isolates UV5 and UV6 show 82% similarity using primer UBC237 (Fig. 5.1), 68% similarity using primer UBC208 (Fig. 5.2), 83% homology using primer UBC228 (Fig. 5.3), 85% homology using primer UBC275 (Fig. 5.4) and 80% homology using primer UBC210 (Fig. 5.6). RAPD primers UBC275 and UBC210 did not show 100% identity between any of the isolates.
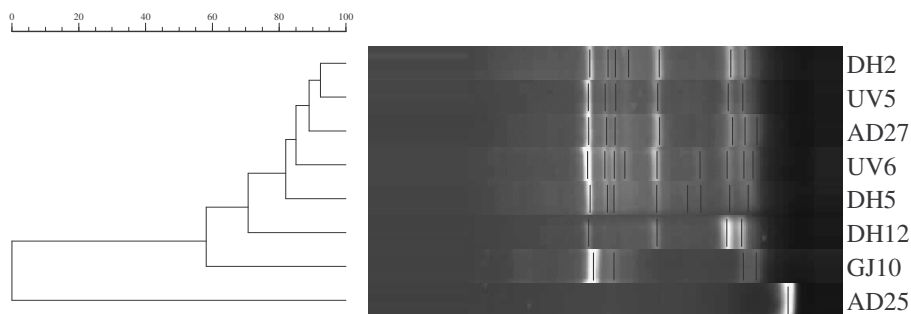
Fig. 5.4:   Dendogram showing the percentage of similarity between isolates based on computer comparisons of RAPD patterns produced using RAPD primer UBC275. *X. autotrophicus* GJ10 was used as the outgroup.
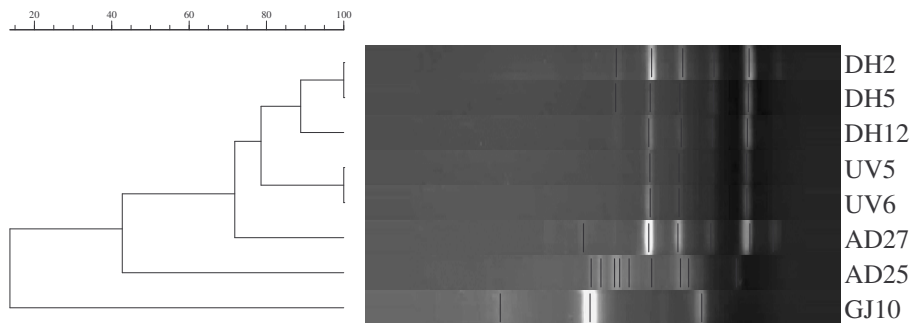
Fig. 5.5: Dendogram showing the percentage of similarity between isolates based on computer comparisons of RAPD patterns produced using RAPD primer UBC245. *X. autotrophicus* GJ10 was used as the outgroup.
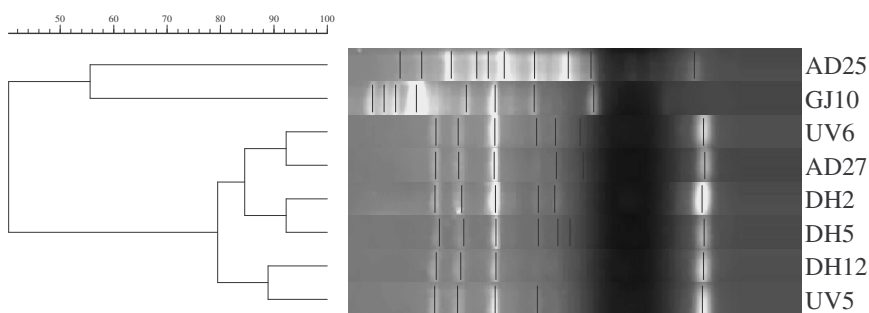
Fig. 5.6: Dendogram showing the percentage of similarity between isolates based on computer comparisons of RAPD patterns produced using RAPD primer UBC210. *X. autotrophicus* GJ10 was used as the outgroup.

5.3.2 PFGE analysis

Restriction of total DNA from isolates DH2 and DH12 with restriction endonuclease *Pst*I (Fig. 5.8) produced identical restriction patterns. These isolates, however, showed an 80% similarity with a *Hin*dIII profile (Fig. 5.7), 100% identity with a *Xho*I profile (Fig.

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5.9), 85% identity with a *Xba*I profile (Fig. 5.10), 88% similarity to a *Cla*I profile (Fig. 5.11) and 84% identity to *Bam*HI profile (Fig. 5.12).
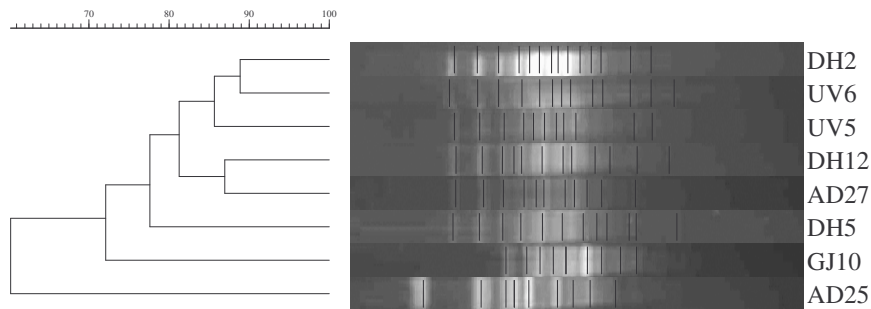
Fig. 5.7:   Dendogram showing the percentage of similarity between isolates based on computer comparisons of PFGE patterns produced using *Hin*dIII digested total DNA. *X. autotrophicus* GJ10 was used as the outgroup.
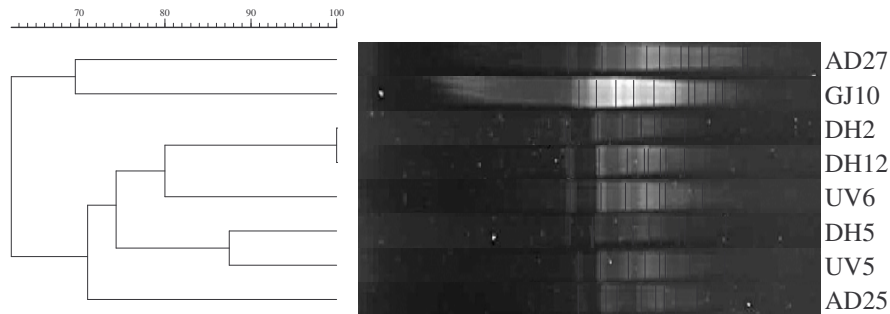
Fig. 5.8:   Dendogram showing the percentage of similarity between isolates based on computer comparisons of PFGE patterns produced using *Pst*I digested total DNA. *X. autotrophicus* GJ10 was used as the outgroup.

Profiles produced using the restriction endonuclease *Xho*I indicated that three isolates, DH2, DH5 and DH12 are 100% identical. These isolates share 78% identity when digested with *Hin*dIII (Fig. 5.7), 74% identity when digested with *Pst*I (Fig. 5.8), 85% identity when digested with *Xba*I (Fig. 5.10), 88% identity when digested with *Cla*I (Fig. 5.11) and 82% identity when digested with *Bam*HI (Fig. 5.12).
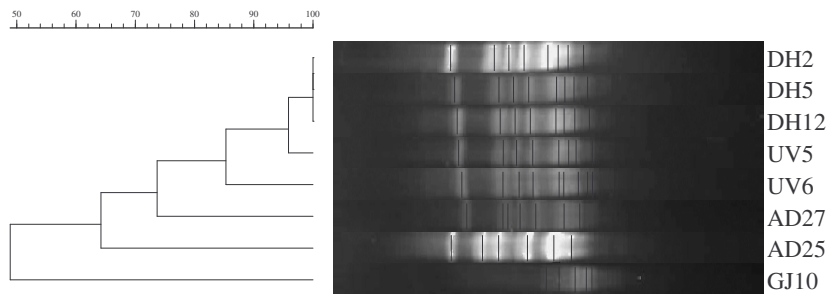
Fig. 5.9: Dendogram showing the percentage of similarity between isolates based on computer comparisons of PFGE patterns produced using *Xho*I digested total DNA. *X. autotrophicus* GJ10 was used as the outgroup.

Three isolates DH2, DH5 and AD27 show 100% identity when digested with *Xba*I. However, these isolates possess 81% similarity when digested with *Hin*dIII (Fig. 5.7), 74% identity when digested with *Xho*I (Fig. 5.9), 88% identity when digested with *Cla*I (Fig. 5.11) and 82% identity when digested with *Bam*HI (Fig. 5.12). None of the restriction enzymes produced identical restriction profiles for all of the *A. aquaticus* isolates.
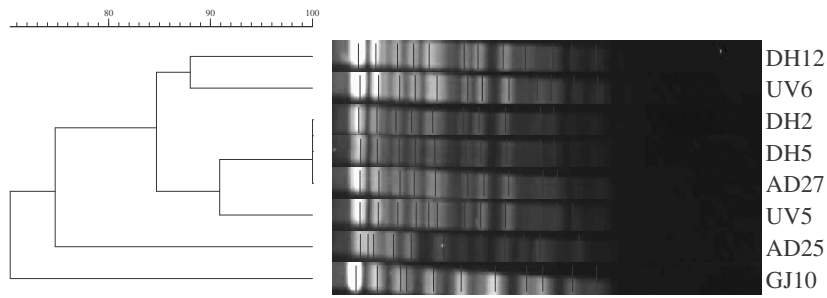
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Fig. 5.10: Dendogram showing the percentage of similarity between isolates based on computer comparisons of PFGE patterns produced using *Xba*I digested total DNA. *X. autotrophicus* GJ10 was used as the outgroup.
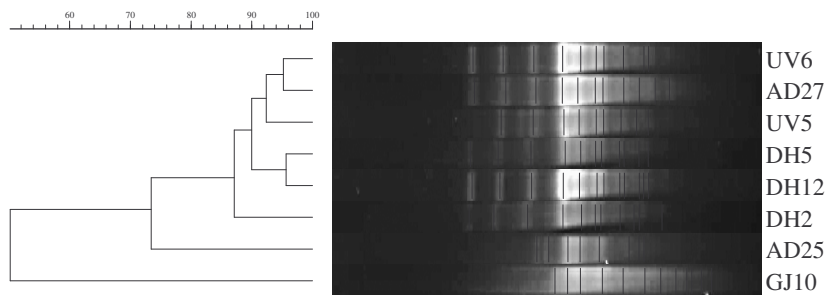
Fig. 5.11: Dendogram showing the percentage of similarity between isolates based on computer comparisons of PFGE patterns produced using *Cla*I digested total DNA. *X. autotrophicus* GJ10 was used as the outgroup.
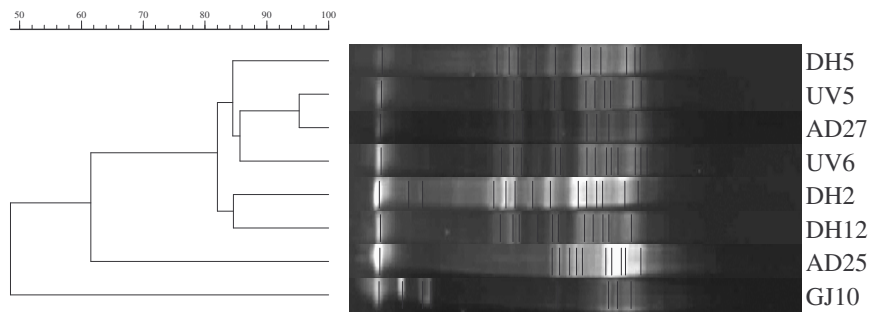
Fig. 5.12: Dendogram showing the percentage of similarity between isolates based on computer comparisons of PFGE patterns produced using *Bam*HI digested total DNA. *X. autotrophicus* GJ10 was used as the outgroup.

5.4   DISCUSSION

The aim of this Chapter was to differentiate between isolates of *A. aquaticus* using PFGE and RAPD analysis. Both PFGE and RAPD analysis reflect the structural organization of bacterial genomes.   PFGE (Schwartz and Cantor, 1984) analyses the chromosomal restriction patterns while RAPD (Welsh and McClelland, 1990; Williams *et al*., 1990) is a PCR based method that compares arbitrary priming sites within bacterial genomes. Both of these bacterial fingerprinting techniques have been extensively used as a means of phylogenetic characterization of bacterial strains (Olive and Bean, 1999). The use of DNA-based typing methods has always been a powerful tool contributing to the understanding of epidemic outbreaks, recurrent infections and clonal dissemination of antimicrobial-resistant bacteria (Sader *et al*., 1995).   In this study PFGE and RAPD analysis was successfully used to differentiate environmental isolates of the haloalkane degrading bacterium *A. aquaticus* isolated from different geographical regions.

The comparison of profiles has been achieved using computer assisted software that constructs dendograms indicating the level relatedness between profiles as percentages. When comparing PFGE profiles Tenover *et al*. (1995) describes isolates as being genetically indistinguishable if their restriction patterns have the same number of bands and the corresponding bands are the same apparent size. Isolates are considered to be closely related if PFGE patterns produce a change consistent with a single genetic event usually a point mutation, or an insertion or deletion of DNA. Spontaneous mutations that create new chromosomal restriction sites will split one restriction fragment into two and

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alteration of a restriction site may result in the absence of a fragment. Generally, isolates are considered to be unrelated if their PFGE patterns differ by changes consistent with three or more independent genetic events (generally seven or more band differences) implying that less than 50% of the well resolved fragments are present in the pattern (Tenover *et al*., 1995).

The incorporation of control strains as well as molecular size standards has always been an important consideration whenever obtaining PFGE profiles (Tenover *et al*., 1995). The use of control organisms affirms that (i) the procedure, including the cell lysis, washing and endonuclease digestion steps are working; (ii) appropriate gel and electrophoretic conditions are used and (iii) the procedure yields results that are reproducible. The use of molecular size standards is generally important in order to determine minor profile differences that may result from single genetic events such as deletions, insertions and mutations (Tenover *et al*., 1995). In this study two previously characterized isolates of *A. aquaticus* (AD25 and AD27) have been used as controls. Molecular size markers have also been used but have been removed for the construction of dendograms as these are not required for phylogenetic analysis.

The comparison of multiple PFGE restriction profiles has always been an important consideration for strain differentiation, as genetic events may not necessarily be restricted to changing a single restriction endonuclease site. Comparison of multiple restriction profiles may provide more useful information as to the level of relatedness between a group of bacterial isolates (Olive and Bean, 1999). Profiles generated using the common

restriction endonucleases *Hin*dIII, *Pst*I, *Xho*I, *Xba*I, *Cla*I and *Bam*HI were compared by the construction of dendograms using a specialized software package. The use of multiple restriction endonucleases proved to be useful as some of the profiles were identical for one restriction endonuclease but different for the other enzymes. No two isolates had identical PFGE restriction endonuclease profiles for all enzymes employed in this study. This indicates that the isolates are so closely related that some of the restriction sites are conserved while others have been mutated to generate a different profile. Profiles generated by restriction endonucleases *Bam*HI (Fig. 5.12), *Cla*I (Fig. 5.11) and *Hin*dIII (Fig. 5.7) did not show 100% similarity between any of the isolates and therefore have the best discriminatory power to differentiate between isolates of *A. aquaticus*.

Isolates DH2 and DH12 had identical restriction profiles for restriction endonucleases *Pst*I (Fig. 5.8) and *Xba*I (Fig. 5.10) but not for any of the other enzymes. None of the other isolates had identical profiles for more than one enzyme. This may indicate that isolates DH2 and DH12 are the most closely related. Isolate DH2 also had an identical *Xba*I restriction profile to isolate DH5 and isolates DH2, DH5 and DH12 had identical *Xho*I restriction profiles. These three isolates are very closely related and this stands to reason as they were all isolated from the same geographic region but from different points in a waste water treatment plant.

The RAPD assay makes use of single short primers with arbitrary nucleotide sequences in a polymerase chain reaction to amplify genomic DNA. This simple and fast technique

produces profiles of amplification products after electrophoretic separation that can be used to fingerprint bacterial strains (Williams *et al.*, 1990). The primers used in this study were of a relatively high mol % (G + C); 70% in the case of UBC237 and 210; and 80% in the case of UBC208, 228, 275 and 245. These were chosen because primers having a mol % (G + C) less than 70% did not produce sufficient banding to yield suitable profiles (data not shown). The ability of these primers to produce multi-band profiles may be due high mol % (G + C) of *A. aquaticus* (Staley *et al.*, 2005).

Analysis of RAPD profiles indicate that isolates DH5 and DH12 are identical based on primer UBC237 (Fig. 5.1) while isolates DH5 and AD27 are identical for primer UBC228 (Fig. 5.3). Isolate UV6 is identical to isolate AD27 based on the profile generated by primer UBC208 (Fig. 5.2). The profile generated for primer UBC245 (Fig. 5.5) indicates that isolates DH2 is identical to DH5 and isolate UV5 is identical to isolate UV6. This indicates that these isolates may be very closely related. Isolates UV5 and UV6 have been isolated from the same geographic region. Isolates DH5 and UV6 appear to be more closely related to the other isolates but show a maximum relationship of 90% shown by the profile of UBC237. Profiles generated by RAPD primers UBC275 (Fig. 5.4) and 210 (Fig. 5.6) did not show 100% identity between any of the isolates and is therefore most suited as markers to differentiate between different isolates of *A. aquaticus*.

The underlying assumption of RAPD analysis is that bands of the same size originate from the same genomic locus thus the production of differing RAPD patterns enables the

comparison of otherwise indistinguishable isolates (Huff *et al*., 1994). RAPD analysis enables the detection of polymorphisms within the amplified DNA fragments that are useful for systematics, population genetics, genome mapping and individual DNA fingerprinting. The RAPD technique is simple, fast and does not require radioactive chemicals. However, it may have several drawbacks. There may be non-reproducibility of the assay because of the dynamics of the amplification procedure (Cipriani *et al*., 1996). A key concern in the use of RAPD technique has been reproducibility, which is greatly influenced by the PCR reaction mixture and amplification conditions which raises concerns as to whether it can be suitably used to generate fingerprints that can be used to differentiate between isolates of *A. aquaticus*.

There is no well defined genetic basis for the polymorphisms generated by RAPD and the presence or absence of a specific band can arise from a point mutation as well as from a mutation event such as insertion or deletion of DNA sequences. The profile obtained is usually due to amplification from unique and repetitive sequences that may evolve at different rates within the genome thus resulting in polymorphisms between isolates (Assigbetse *et al*., 1994). The main advantages of RAPD analysis are (i) suitability for work on anonymous genomes, (ii) applicability to problems where only limited quantities of DNA are available (iii) efficiency and low expense (Hadrys *et al*., 1992).

Molecular typing methods have predominantly been used in recent times for strain differentiation in epidemiological studies because it is commonly assumed that outbreaks of infectious disease often result from exposure to a common etiological agent (Olive and

Bean, 1999). These methods have also been extended to differentiate industrially important bacteria such as Lactobacilli (Tynkkynen *et al*., 1999) and Pediococci (Simpson *et al*., 2002). Several studies have compared the relative efficiency of the PFGE and RAPD techniques as tools for the typing bacterial isolates (Saulnier *et al*., 1993; Barbier *et al*., 1996; Tynkkynen *et al*., 1999; Simpson *et al*., 2002), each weighing out the relative advantages and disadvantages of the techniques as the results obtained using these molecular typing methods cannot be directly compared.

In this study both PFGE and RAPD analysis revealed a suitable amount of genetic diversity among isolates of *A. aquaticus* from different sources thus making both PFGE and RAPD analysis suitable methods for the typing of new haloalkane degrading isolates of *A. aquaticus*. The outcomes of these molecular fingerprinting techniques are not eh same as RAPD is a PCR-based approach using arbitrary primers and PFGE involves the use the use of restriction endonucleases that cleave the DNA at specific loci. It was however, found that the PFGE technique showed a greater amount of polymorphisms between the isolates that were tested and may thus be the method of choice when typing new isolates of *Ancylobacter* from different geographic regions.

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CHAPTER SIX

GENERAL DISCUSSION

6.1   THE RESEARCH IN PERSPECTIVE

With the dawn of the industrial revolution there has been a global increase in the use of industrially produced chlorine-based compounds, many of which are extremely toxic to the environment. Many of these compounds have found their way into terrestrial and aquatic ecosystems mainly due to improper disposal practices, poor handling and accidental spillages (Fetzner, 1998; De Wildeman and Verstraete, 2003).   The halogenated hydrocarbon, 1,2-dichloroethane (DCA), is the most frequently used and produced industrial solvent. The recalcitrance of this compound within the environment has become a major concern in recent times due to its solubility in water which contributes to its dissemination within aquatic ecosystems (Janssen *et al*., 2005).

The remediation of contaminated soil and water has become of primary importance and is currently restricted to mechanical, chemical or biological remediation strategies. Mechanical and chemical treatment methods are generally more expensive and difficult to implement. Bioremediation offers an alternative safer and cheaper strategy of cleaning up contaminated sites.   The introduction of synthetic chemicals with structures and properties to which microorganisms have not been exposed to during the course of evolution, have created new opportunities for microorganisms to evolve metabolic pathways in order to exploit new growth substrates or detoxify harmful chemicals. Thus

far, several bacterial isolates have been identified that possess the enzymatic potential to cleave the carbon-halogen bond in halogenated hydrocarbons. Janssen *et al*. (2005), provides a complete review of some bacterial mechanisms of xenobiotic degradation.

The present study focused on the isolation and characterization of new bacteria that possess the ability to completely mineralize DCA. These bacteria were preliminarily identified and the dehalogenase gene involved in cleaving the first carbon-halogen bond was identified. Attempts were made to identify any of the other genes that may be involved in the complete mineralization of DCA. Biochemical assays were also performed in order to determine whether the DCA catabolic route was similar to that of *X. autotrophicus* GJ10. Finally, DNA profiling techniques such RAPD and PFGE were used to determine the level of similarity between isolates from different geographic regions.

In order to isolate bacteria capable of utilizing DCA as a carbon and energy source, waste water facilities handling chlorinated compounds had to be identified. The Northern Wastewater Works located in Durban, KwaZulu-Natal which processes large quantities of industrial waste water was chosen as the first site from which samples could be obtained. The second site chosen was the SAPPI (South African Pulp and Paper Industry) plant which commonly utilizes bleach in the paper whitening process and treats waste water in an in-house treatment plant. Bacterial isolates obtained from these sites were batch cultured into a minimal medium containing 5mM DCA as sole carbon source thus selecting only for bacteria capable of DCA utilization. These isolate were then

screened for dehalogenase activity using a halide release assay. A total of 187 isolates were screened and of these five (DH2, DH5, DH12, UV5 and UV6) were positive for dehalogenase activity. These isolates were identified by 16S rDNA sequencing and found to be different strains of *Ancylobacter aquaticus* (Appendix A). The isolation method described here may be extended and routinely used to isolate other culturable xenobiotic degrading microorganisms from any possible source.

The molecular fingerprinting techniques, RAPD and PFGE indicated that none of the isolates were identical on a phylogenetic level. RAPD analysis was able to show that some of the isolates were identical using one primer but were different when another primer was used. The separation of restricted DNA using PFGE indicated that although some of the isolates had identical profiles when restricted with one enzyme, a different profile for these isolates was obtained when another enzyme was used. The PFGE technique was however, found to be a better technique as a greater number of polymorphisms could be observed. Both of these techniques are suitable for typing strains of *A. aquaticus* with choice of primer or restriction endonuclease being important for the relevant technique. The use of molecular typing methods show that the South African isolates of *Ancylobacter* are different from each other and the previously characterized isolates indicating diversity within this group of degradative bacteria.

The PCR primers used in this study were designed based on known dehalogenase gene sequences and revealed that all of the South African isolates had a common dehalogenase gene (*dhlA*). This gene had been first characterized in *X. autotrophicus* GJ10 (Janssen *et*

al., 1989) and later in isolates of *A. aquaticus* (van den Wijngaard *et al*., 1992). Recently, Song *et al*. (2004) isolated *X. flavus* UE15 that possessed a similar hydrolytic dehalogenase gene (Fig. 6.1). Alignment of gene sequences indicated base substitutions with no change in the overall translated sequence (Appendix B). These primers that have been designed based on conserved regions of known dehalogenases may be used to identify identical or closely related genes in other bacterial isolates capable of halogenated hydrocarbon degradation. This is the first hydrolytic dehalogenase gene to be identified in DCA degrading bacterial isolates from Southern Africa which has relatively recently been exposed to DCA pollution. The presence and identification of the *dhlA* gene in these isolates indicates a global distribution of this key gene in both isolates of *Xanthobacter* and *Ancylobacter*.

The oxidation of 2-chloroethanol to chloroacetaldehyde (Fig. 6.1) was found to much lower in all isolates compared to *X. autotrophicus* GJ10 (Janssen *et al*., 1987) and the previously characterized isolates of *A. aquaticus* (van den Wijngaard *et al*., 1992). The chloroethanol dehydrogenase of *X. autotrophicus* GJ10 appeared to be a PQQ-containing alcohol dehydrogenase that due its broad specificity was active with 2-chloroethanol. This alcohol dehydrogenase found in isolates of *A. aquaticus* may also be a quinoprotein containing alcohol dehydrogenase as these have been identified in several methylotrophic bacteria (Janssen *et al*., 1987).

There are two homologs of the aldehyde dehydrogenase gene (*aldA* and *aldB*) present in *X. autotrophicus* GJ10 (Bergeron *et al*., 1998) which are involved in the conversion of 2-chloroacetaldehyde to monochloroacetic acid (Fig. 6.1). Primers designed for these 84%

similar genes could only amplify the *aldB* gene of *X. autotrophicus* GJ10 which was subsequently used as probe after digoxygenin labeling. The probe was found to not only hybridize to the correct regions of *Sma*I digested *X. autotrophicus* GJ10 DNA but also hybridized to DNA of the *A. aquaticus* isolates but in different regions. This indicates that all of the *A. aquaticus* isolates may possess an aldehyde dehydrogenase homolog.
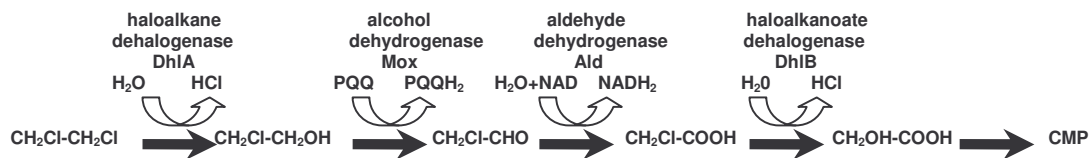
Fig. 6.1: Catabolic route for the metabolism of DCA in *X. autotrophicus* GJ10, CMP (central metabolic pathway), (Adapted from Janssen *et al*., 1985).

Primers designed for a transposase first identified downstream of the *dhlB* gene (haloacid dehalogenase) of *X. autotrophicus* GJ10 (van der Ploeg *et al*., 1991) and later upstream to the *dlhA* gene of *X. flavus* UE15 (Song *et al*., 2004) was able to amplify the gene in isolates DH12, UV6, AD27 and *X. autotrophicus* GJ10. When the amplified gene from *X. autotrophicus* GJ10 was used as a probe, multiple signals were obtained for all isolates indicating that multiple homologs for this gene may be present in all of the isolates including *X. autotrophicus* GJ10. The primers designed for the haloacid dehalogenase (*dhlB*) could only amplify the gene in *X. autotrophicus* GJ10 and none of the other isolates. However, when used as labeled probe against *Bcl*I digested DNA hybridization signals were obtained for *A. aquaticus* isolates DH12, UV5 and AD27, indicating that

homologs may be present in these isolates. The presence of aldehyde dehydrogenase and haloacid dehalogenase homologs indicates that these isolates may have acquired similar genes involved in the catabolism of DCA. The diversity in aldehyde dehydrogenase and haloacid dehalogenase sequences may reflect the natural occurrence of aldehydes and haloacids in nature. The aldehyde dehydrogenase and haloacid dehalogenase genes are probably of ancient evolutionary origin and their widespread distribution may not be due to environmental contamination.

In order to determine whether the catabolic route of DCA degradation among the South African isolates is similar to *X. autotrophicus* GJ10 as well as previously characterized isolates of *A. aquaticus*, intermediates in the pathway (Fig. 6.1) were used as substrates in enzymatic reactions involving crude cell lysates from each of the isolates. Quantitative enzyme assays indicated all of the isolates possessed varying activities to the different substrates in the DCA catabolic pathway. This suggests that the first gene (*dhlA*) involved in degradation of DCA is globally distributed even though exposure to DCA in the Southern Africa is recent. The migration of DCA degrading bacteria in the ballast tank of ships may be an alternative explanation as to how this pathway is present in Southern African isolates.

The second part of the study involved ascertaining whether the genes involved in the mineralization of DCA were present and located in similar regions within the genome of these DCA degrading bacteria. Total DNA isolated from all isolates including *X. autotrophicus* GJ10 were restricted with different restriction endonucleases and

transferred onto nylon membranes by Southern transfer. Digoxygenin labeled probes were prepared by labeling PCR amplified genes of *X. autotrophicus* GJ10 involved in DCA degradation. Hybridization signals using the *dhlA* labeled probe indicated that all of the isolates possessed the *dhlA* gene on a common 8kb *Eco*RI restriction fragment. In *X. autotrophicus* GJ10 the *dhlA* gene is found on a 200kb plasmid, pXAU1 which may also be present in isolates of *A. aquaticus*. The *dhlA* gene may have evolved in either genus and may have transferred to the other genus by conjugation or mobilization of the plasmid where it was located; thus resulting in an identical gene been present in two entirely different genera. This is generally the method by which genes become distributed within bacterial communities.

The current study was successful in isolating and identifying DCA degrading bacteria from the Southern Africa. The pathway and first gene (*dhlA*) involved in DCA degradation has been elucidated and shown to be similar to the previously characterized DCA degrading bacteria indicating a global distribution of the DCA catabolic gene (*dhlA*). Molecular typing methods have been able to show that these isolates are distinguishable from each as well as two previously characterized isolates. This study forms the basis for the development and use of these bacterial isolates for future bioremediation strategies as well as an understanding of how genes involved in xenobiotic degradation become globally distributed.

The global distribution of catabolic genes between different genera of bacteria may be attributed to several physical and biological mechanisms. Bacteria can be removed from

their ecological niches and distributed throughout the planet in just about any physical way. The migration of people between continents may be the most influential physical method by which bacteria have been spread to the different continents and this best noted with the spread of pathogenic bacteria. Other physical methods include wind and water. Ships carrying sea water in the ballasts have been found to contribute significantly to the spread of bacteria between continents (Drake *et al*., 2007). It has also been found that some bacteria possess the ability to colonise clouds and can thus travel great distances (Everts, 2008). The widespread transfer of catabolic bacteria and genes may not necessarily be limited to environmental contamination but rather the transfer of bacteria to new ecological niches where they possess the inherent capacity to proliferate by degrading or utilizing contaminants as carbon and energy sources.

The transfer of genes to other bacteria may be more restricted to transformation, conjugation and bacteriophage mediated transduction. Many bacterial species are capable of acquiring DNA from their immediate environment and integrating this DNA into their genomes. This DNA may contain catabolic genes and results in transformation of the bacterium to develop novel functions which may improve its survival in contaminated environments.

A frequently encountered method of gene acquisition is generally through conjugation involving conjugative or mobilisable plasmids carrying catabolic genes. These plasmids are able to cross genera to different hosts thus conferring novel phenotypes to their recipients. Entire operons and regulons carrying catabolic genes may be transferred to

new hosts. The expression of the catabolic genes may be regulated by the plasmids and expression of enzymes involved in the degradation of xenobiotic compounds may be initiated almost immediately (del la Cruz and Davis, 2000).

The first gene, *dhlA,* which codes for the hydrolytic dehalogenase in the DCA catabolic route of *X. autotrophicus* GJ10 is located on a large plasmid, pXAU1. Due to a lack of a suitable selection marker that can be used during conjugation experiments, it could not be determined whether pXAU1 is conjugative or mobilisable (Tardif *et al*., 1991). This raises the question as to how a homologous *dhlA* gene could be found in *X. autotrophicus* GJ10 (Janssen *et al*., 1985), *X. fluvus* UE15 (Song *et al*., 2004), *A. aquaticus* AD25 and AD27 (van den Wijngaard *et al*., 1992) as well as the South African isolates of *Ancylobacter*.

One possible explanation may be that pXAU1 is indeed conjugative as not all possible selective markers have been screened or that pXAU1 is mobilisable and requires a conjugative plasmid partner to move to between hosts (Tardif *et al*., 1999). Conjugative or mobilisable plasmids lacking selective markers have been found to disseminate between hosts undetected (del la Cruz and Davies, 2000) and this may be the most likely method by which the *dhlA* gene has become globally distributed.

Lysogenic bacteriophages found commonly in terrestrial and aquatic ecosystems may also influence the transfer of catabolic genes from one host to another. During integration into the catabolic host chromosome these bacteriophages may inadvertently

pick up catabolic genes when reverting back to the lytic cycle. Upon lysis of the host progeny bacteriophages may integrate these catabolic genes into the genome of the new host upon infection. This method of gene transfer occurs very frequently in nature and has been implicated in transfer of catabolic genes involved in degradation of pesticides (Ghosh *et al.*, 2008)

Genes or gene clusters that are ubiquitously found on various chromosomes, plasmids and phages often reside on the discrete mobile, but not self-replicative, elements represented by transposons, integrons and pathogenicity islands (Tsuda *et al.*, 1999). An insertion element IS*1247* which may be located on a transposon has been identified in *X. autotrophicus* GJ10 which appears to influence the expression of the haloacetate gene *dhlB* (van der Ploeg *et al.*, 1995). Transposons may be defined as discrete DNA segments that can move from one site to another without the need for genetic homology between the donor and target sites. A transposase encoded by the transposon catalyses not only the specific single- or double stranded cleavage of the transposon ends at the donor site but also the non-specific double-stranded cleavage of the target site (Hallet and Sherratt (1997). Transposons carrying catabolic genes thus have the ability to translocate between the chromosome and a plasmid and if the plasmid is conjugative the catabolic can be transferred to new hosts thus conferring new catabolic properties (Tsuda *et al.*, 1999).

The presence of similar catabolic genes within different genera of bacteria is not restricted to *dhlA* gene of *X. autotrophicus* GJ10. The widespread use of the herbicide

2,4-dichlorophenoxyacetic acid (2,4-D) across the different continents has resulted in the global dissemination of various (2,4-D) catabolic genes (McGowan *et al*., 1998). Microbes comprise the most diverse forms of life as they represent the richest repertoire of molecular and chemical diversity in nature. Their existence in extremely diverse environments has led to the evolution of an extensive range of enzymes and metabolic pathways that enables their survival in even the harshest of conditions. Microorganisms may be regarded as nature's original recyclers often converting toxic organic compounds into harmless products and scientists have began harnessing this potential to transform or degrade xenobiotics by isolating microorganisms from various sources particularly contaminated sites or treatment facilities handling xenobiotics (Jain *et al*., 2005)

6.2   POTENTIAL FOR FUTURE DEVELOPMENT OF THE WORK

The industrial uses of chlorinated hydrocarbons especially 1,2-dichloroethane (DCA) has increased significantly in recent years and this has lead to an increase in global production. This highly toxic compound has entered terrestrial and aquatic ecosystems mainly due to poor handling, accidental spillages and inappropriate disposal practices (De Wildeman and Verstraete, 2003). Of the various remediation strategies that are currently available bioremediation is the cheapest and easiest to implement. The ability of microorganisms to evolve novel catabolic routes that can completely mineralize certain toxic compounds makes them the ideal candidates for any bioremediation strategy (Copley, 2000).

The procedure described in Chapter Two of this study to isolate any culturable bacterium capable of DCA degradation can be extended to include any halogenated hydrocarbon. The widespread use of chlorinated compounds in both agriculture and industry has resulted in bacteria evolving and acquiring a vast repertoire of genes that enable them to adapt to the presence of these compounds. The procedure is simple to perform and can be implemented in any bio-augmentation strategy to enrich for only bacteria involved in the remediation process. In this process, bacteria involved in biodegradation can be isolated from contaminated sites and reintroduced into contaminated environment to enhance the remediation process.

At present the bacteria capable of DCA degradation can be grouped into two different groups based on nutritional requirements. *X. autotrophicus* GJ10 (Janssen *et al*., 1985), *X. fluvus* UE15 (Song *et al*., 2004) and the different isolates of *A. aquaticus* (van den Wijngaard *et al*., 1992) have a requirement for various vitamins and co-factors and can thus be grouped together while *Pseudomonas* sp strain DE2 (Stucki *et al*., 1983) and *Pseudomonas* sp strain DCA1 (Hage and Hartmans, 1999) do not have a requirement for vitamins and co-factors. Bioremediation strategies involving the use of bacterial isolates that require additional nutrients may not necessarily be cost-effect for *in situ* or *ex situ* treatment of DCA contaminated sites. Bacterial isolates such a *Pseudomonas* sp strain DE2 and *Pseudomonas* sp strain DCA1 were found to also have a higher substrate (DCA) affinity as compared to *X. autotrophicus* GJ10 (Hage and Hartmans, 1999), thus the use of these bacterial isolates would be more cost effective when used to treat DCA contamination.

Recently, the DCA catabolic pathway of *X. autotrophicus* GJ10 has been transferred to plants and is now being implemented in various phytoremediation strategies (Mena-Benitez *et al*., 2008). The current study forms the basis in the understanding of the genes involved in DCA degradation by isolates of *A. aquaticus*. Further research is required in characterization of the aldehyde dehydrogenase and haloacid dehalogenase of *A. aquaticus*. These enzymes are pivotal in the DCA catabolic route of *A. aquaticus* their uses may be extended to chemical and pharmaceutical industries to generate new enantiopure chemicals.

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APPENDIX:

Appendix A: Alignment of 16S rDNA gene sequences of five newly isolated dehalogenase producers with *A. aquaticus* AD27. Conserved regions are darkly shaded while non-conserved regions appear lighter.

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              *        20         *        40         *        60         *        80
AD27:  GGGCGGTGTGTACAAGGCCCGGGtAACGTATTCACCGTGGCATGCTGATCCACGATTACTAGCGATTCCAACTTCATGCACTCGAGT :  87
DH2 :  GGGCGGTGTGTACAAGGCCCGGGtAACGTATTCACCGTGGCATGCTGATCCACGATTACTAGCGATTCCAACTTCATGCACTCGAGT :  87
DH12:  GGGCGGTGTGTACAAGGCCCGGGAAACGTATTCACCGTGGCATGCTGATCCACGATTACTAGCGATTCCAACTTCATGCACTCGAGT :  87
DH5 :  GGGCGGAGTGTACAAGGCCCGGGtAACGTATTCACCGTGGCATGCTGATCCACGATTACTAGCGATTCCAACTTCATGCACTCGAGT :  87
UV5 :  GGGCGGTGTGTACAAGGCCCGGGtAACGTATTCACCGTGGCATGCTGATCCACGATTACTAGCGATTCCAACTTCATGCACTCGAGT :  87
UV6 :  GGGCGGTGTGTACAAGGCCCGGGtAACGTATTCACCGTGGCATGCTGATCCACGATTACTAGCGATTCCAACTTCATGCACTCGAGT :  87
       GGGCGGtGTGTACAAGGCCCGGGtAACGTATTCACCGTGGCATGCTGATCCACGATTACTAGCGATTCCAACTTCATGCACTCGAGT

              *       100         *       120         *       140         *       160         *
AD27:  TGCAGAGTGCAATCCGAACTGAGACGGCTTTTGGAGATTTGCTTACCCTCGCGGGtTCGCTTCCCACTGTCACCGCCATTGTAGCAC : 174
DH2 :  TGCAGAGTGCAATCCGAACTGAGACGGCTTTTGGAGATTTGCTTACCCTCGCGGGtTCGCTTCCCACTGTCACCGCCATTGTAGCAC : 174
DH12:  TGCAGAGTGCAATCCGAACTGAGACGGCTTTTGGAGATTTGCTTACCCTCGCGGGtTCGCTTCCCACTGTCACCGCCATTGTAGCAC : 174
DH5 :  TGCAGAGTGCAATCCGAACTGAGACGGCTTTTGGAGATTTGCTTACCCTCGCGGGCTCGCTTCCCACTGTCACCGCCATTGTAGCAC : 174
UV5 :  TGCAGAGTGCAATCCGAACTGAGACGGCTTTTGGAGATTTGCTTACCCTCGCGGGtTCGCTTCCCACTGTCACCGCCATTGTAGCAC : 174
UV6 :  TGCAGAGTGCAATCCGAACTGAGACGGCTTTTGGAGATTTGCTTACCCTCGCGGGtTCGCTTCCCACTGTCACCGCCATTGTAGCAC : 174
       TGCAGAGTGCAATCCGAACTGAGACGGCTTTTGGAGATTTGCTTACCCTCGCGGGtTCGCTTCCCACTGTCACCGCCATTGTAGCAC

              180         *       200         *       220         *       240         *       260
AD27:  GTGTGTAGCCCAGCCCGTAAGGGCCATGAGGACTTGACGTCATCCCCACCTTCCTCGCGGCTTATCACCGGCAGTCCCCCTAGAGTG : 261
DH2 :  GTGTGTAGCCCAGCCCGTAAGGGCCATGAGGACTTGACGTCATCCCCACCTTCCTCGCGGCTTATCACCGGCAGTCCCCCTAGAGTG : 261
DH12:  GTGTGTAGCCCAGCCCGTAAGGGCCATGAGGACTTGACGTCATCCCCACCTTCCTCGCGGCTTATCACCGGCAGTCCCCCTAGAGTG : 261
DH5 :  GTGTGTAGCCCAGCCCGTAAGGGCCATGAGGACTTGACGTCATCCCCACCTTCCTCGCGGCTTATCACCGGCAGTCCCCCTAGAGTG : 261
UV5 :  GTGTGTAGCCCAGCCCGTAAGGGCCATGAGGACTTGACGTCATCCCCACCTTCCTCGCGGCTTATCACCGGCAGTCCCCCTAGAGTG : 261
UV6 :  GTGTGTAGCCCAGCCCGTAAGGGCCATGAGGACTTGACGTCATCCCCACCTTCCTCGCGGCTTATCACCGGCAGTCCCCCTAGAGTG : 261
       GTGTGTAGCCCAGCCCGTAAGGGCCATGAGGACTTGACGTCATCCCCACCTTCCTCGCGGCTTATCACCGGCAGTCCCCCTAGAGTG

              *       280         *       300         *       320         *       340
AD27:  CCCAACTTAATGATGGCAACTAAGGGCGAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCGCACGACACGAGCTGACGACAGCC : 348
DH2 :  CCCAACTTAATGATGGCAACTAAGGGCGAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCtCACGACACGAGCTGACGACAGCC : 348
DH12:  CCCAACTTAATGATGGCAACTAAGGGCGAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCtCACGACACGAGCTGACGACAGCC : 348
DH5 :  CCCAACTTAATGATGGCAACTAAGGGCGAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCtCACGACACGAGCTGACGACAGCC : 348
UV5 :  CCCAACTTAATGATGGCAACTAAGGGCGAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCtCACGACACGAGCTGACGACAGCC : 348
UV6 :  CCCAACTTAATGATGGCAACTAAGGGCGAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCtCACGACACGAGCTGACGACAGCC : 348
       CCCAACTTAATGATGGCAACTAAGGGCGAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCtCACGACACGAGCTGACGACAGCC

              *       360         *       380         *       400         *       420         *
AD27:  ATGCAGCACCTGTGTTCCCGGCTCCGAAGAGAAGAAACCATCTCTGGTAACCGTCCGGGACATGTCAAAGGCTGGTAAGGTTCTGCG : 435
DH2 :  ATGCAGCACCTGTGTTCCCGGCTCCGAAGAGAAGAAACCATCTCTGGTAACCGTCCGGGACATGTCAAAGGCTGGTAAGGTTCTGCG : 435
DH12:  ATGCAGCACCTGTGTTCCCGGCTCCGAAGAGAAGAAACCATCTCTGGTAACCGTCCGGGACATGTCAAAGGCTGGTAAGGTTCTGCG : 435
DH5 :  ATGCAGCACCTGTGTTCCCGGCTCCGAAGAGAAGAAACCATCTCTGGTAACCGTCCGGGACATGTCAAAGGCTGGTAAGGTTCTGCG : 435
UV5 :  ATGCAGCACCTGTGTTCCCGGCTCCGAAGAGAAGAAACCATCTCTGGTAACCGTCCGGGACATGTCAAAGGCTGGTAAGGTTCTGCG : 435
UV6 :  ATGCAGCACCTGTGTTCCCGGCTCCGAAGAGAAGAAACCATCTCTGGTAACCGTCCGGGACATGTCAAAGGCTGGTAAGGTTCTGCG : 435
       ATGCAGCACCTGTGTTCCCGGCTCCGAAGAGAAGAAACCATCTCTGGTAACCGTCCGGGACATGTCAAAGGCTGGTAAGGTTCTGCG

              440         *       460         *       480         *       500         *       520
AD27:  CGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCtTTGAGTTTTAATCTTGCGACCGtACTCCCC : 522
DH2 :  CGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCtTTGAGTTTTAATCTTGCGACCGtACTCCCC : 522
DH12:  CGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCtTTGAGTTTTAATCTTGCGACCGtACTCCCC : 522
DH5 :  CGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCtTTGAGTTTTAATCTTGCGACCGCACTCCCC : 522
UV5 :  CGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCtTTGAGTTTTAATCTTGCGACCGtACTCCCC : 522
UV6 :  CGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCGtAtGAGTTTTAATCTTGCGACCGtACTCCCC : 522
       CGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCcTtTGAGTTTTAATCTTGCGACCGtACTCCCC

              *       540         *       560         *       580         *       600
AD27:  AGGCGGGAGGCTTAATGCGTTAGCTGCGCCACTGATGAGCATGCTCACCAACGGCTAGCCTCCATCGTTTACGGCGTGGACTACCAG : 609
DH2 :  AGGCGGGAGGCTTAATGCGTTAGCTGCGCCACTGATGAGCATGCTCACCAACGGCTAGCCTCCATCGTTTACGGCGTGGACTACCAG : 609
DH12:  AGGCGGGAGGCTTAATGCGTTAGCTGCGCCACTGATGAGCATGCTCACCAACGGCTAGCCTCCATCGTTTACGGCGTGGACTACCAG : 609
DH5 :  AGGCGGGAGGCTTAATGCGTTAGCTGCGCCACTGATGAGCATGCTCACCAACGGCTAGCCTCCATCGTTTACGGCGTGGACTACCAG : 609
UV5 :  AGGCGGGAGGCTTAATGCGTTAGCTGCGCCACTGATGAGCATGCTCACCAACGGCTAGCCTCCATCGTTTACGGCGTGGACTACCAG : 609
UV6 :  AGGCGGGAGGCTTAATGCGTTAGCTGCGCCACTGATGAGCATGCTCACCAACGGCTAGCCTCCATCGTTTACGGCGTGGACTACCG : 609
       AGGCGGGAGGCTTAATGCGTTAGCTGCGCCACTGATGAGCATGCTCaCCAACGGCTAGCCTCCATCGTTTACGGCGTGGACTACCaG

              *       620         *       640         *       660         *       680         *
AD27:  GGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGCGTCAGTACCGGACCAGTAAGCCGCCTTCGCCACTGGTGTTCTTGC : 696
DH2 :  GGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGCGTCAGTACCGGACCAGTAAGCCGCCTTCGCCACTGGTGTTCTTGC : 696
DH12:  GGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGCGTCAGTACCGGACCAGTAAGCCGCCTTCGCCACTGGTGTTCTTGC : 696
DH5 :  GGTATCTAATCCTGTTTGCTGCCCCACGCTTTCGCACCTCAGCGTCAGTACCGGACCAGTAAGCCGCCTTCGCCACTGGTGTTCTTGC : 695
UV5 :  GGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGCGTCAGTACCGGACCAGTAAGCCGCCTTCGCCACTGGTGTTCTTGC : 696
UV6 :  GGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGCGTCAGTACCGGACCAGTAAGCCGCCTTCGCCACTGGTGTTCTTGC : 696
       GGTATCTAATCCTGTTTGCTcCCCACGCTTTCGCACCTCAGCGTCAGTACCGGACCAGTAAGCCGCCTTCGCCACTGGTGTTCTTGC

              700         *       720         *       740         *       760         *       780
AD27:  GAATATCTACGAATTTCACCTCTACACTCGCAGTTCCACTTACCTCTTCCCGGACTCGAGATACCCAGTATCAAGGGCAGTTCTGGAG : 783
DH2 :  GAATATCTACGAATTTCACCTCTACACTCGCAGTTCCACTTACCTCTTCCCGGACTCGAGATACCCAGTATCAAGGGCAGTTCTGGAG : 783
DH12:  GAATATCTACGAATTTCACCTCTACACTCGCAGTTCCACTTACCTCTTCCCGGACTCGAGATACCCAGTATCAAGGGCAGTTCTGGAG : 783
DH5 :  GAATATCTACGAATTTCACCTCTACACTCGCAGTTCCACTTACCTCTTCCCGGACTCGAGATACCCAGTATCAAGGGCAGTTCTGGAG : 782
UV5 :  GAATATCTACGAATTTCACCTCTACACTCGCGGTTCCACTTACCTCTTCCCGGACTCGAGATACCCAGTATCAAGGGCAGTTCTGGAG : 783
UV6 :  GAATATCTACGAATTTCACCTCTACACTCGCAGTTCCACTTACCTCTTCCCGGACTCGAGATACCCAGTATCAAGGGCAGTTCTGGAG : 783
       GAATATCTACGAATTTCACCTCTACACTCGCaGTTCCACTTACCTCTTCCCGGACTCGAGATACCCAGTATCAAGGGCAGTTCTGGAG
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           *        800         *        820         *        840         *        860         *
AD27: TTGAGCTCCAGGCTTTCACCCCTGACTTAAATATCCGCCTACGTGCGCTTTACGCCCAGTGATTCCGAACAACGCTAGCCCCCTTCG :  870
DH2 : TTGAGCTCCGGGCTTTCACCCCTGACTTAAATATCCGCCTACGTGCGCTTTACGCCCAGTGATTCCGAACAACGCTAGCCCCCTTCG :  870
DH12: TTGAGCTCCAGGCTTTCACCCCTGACTTAAATATCCGCCTACGTGCGCTTTACGCCCAGTGATTCCGAACAACGCTAGCCCCCTTCG :  870
DH5 : TTGAGCTCCAGGCTTTCACCCCTGACTTAAATATCCGCCTACGTGCGCTTTACGCCCAGTGATTCCGAACAACGCTAGCCCCCTTCG :  869
UV5 : TTGAGCTCCAGGCTTTCACCCCTGACTTAAATATCCGCCTACGTGCGCTTTACGCCCAGTGATTCCGAACAACGCTAGCCCCCTTCG :  870
UV6 : TTGAGCTCCAGGCTTTCACCCCTGACTTAAATATCCGCCTACGTGCGCTTTACGCCCAGTGATTCCGAACAACGCTAGCCCCCTTCG :  870
      TTGAGCTCCaGGCTTTCACCCCTGACTTAAATATCCGCCTACGTGCGCTTTACGCCCAGTGATTCCGAACAACGCTAGCCCCCTTCG

          880         *        900         *        920         *        940         *
AD27: TATTACCGCGGCTGCTGGCACGAAGTTAGCCGGGGCTTCTTCTCCGACTACCGTCATTATCTTCGTCGGCGCAAGAGCTTTACAACC :  957
DH2 : TATTACCGCGGCTGCTGGCACGAAGTTAGCCGGGGCTTCTTCTCCGACTACCGTCATTATCTTCGTCGGCGAAAGAGCTTTACAACC :  957
DH12: TATTACCGCGGCTGCTGGCACGAAGTTAGCCGGGGCTTCTTCTCCGACTACCGTCATTATCTTCGTCGGCGAAAGAGCTTTACAACC :  957
DH5 : TATTACCGCGGCTGCTGGCACGAAGTTAGCCGGGGCTTCTTCTCCGACTACCGTCATTATCTTCGTCGGCGAAAGAGCTTTACAACC :  956
UV5 : TATTACCGCGGCTGCTGGCACGAAGTTAGCCGGGGCTTCTTCTCCGACTACCGTCATTATCTTCGTCGGCGAAAGAGCTTTACAACC :  957
UV6 : TATTACCGCGGCTGCTGGCACGAAGTTAGCCGGGGCTTCTTCTCCGACTACCGTCATTATCTTCGTCGGCGAAAGAGCTTTACAACC :  957
      TATTACCGCGGCTGCTGGCACGAAGTTAGCCGGGGCTTCTTCTCCGACTACCGTCATTATCTTCGTCGGCGaAAGAGCTTTACAACC

      960         *        980         *       1000         *       1020         *       1040
AD27: CTAAGGCCTTCATCACTCACGCGGCATGGCTGGATCAGGCTTGCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTC : 1044
DH2 : CTAAGGCCTTCATCACTCACGCGGCATGGCTGGATCAGGCTTGCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTC : 1044
DH12: CTAAGGCCTTCATCACTCACGCGGCATGGCTGGATCAGGCTTGCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTC : 1044
DH5 : CTAAGGCCTTCATCACTCACGCGGCATGGCTGGATCAGGCTTGCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTC : 1043
UV5 : CTAAGGCCTTCATCACTCACGCGGCATGGCTGGATCAGGCTTGCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTC : 1044
UV6 : CTAAGGCCTTCATCACTCACGCGGCATGGCTGGATCAGGCTTGCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTC : 1044
      CTAAGGCCTTCATCACTCACGCGGCATGGCTGGATCAGGCTTGCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTC

           *       1060         *       1080         *       1100         *       1120         *
AD27: TGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCTCAGACCAGCTACGGATCGTCGCCTTGGTGCGCCTTTACCACACCAACT : 1131
DH2 : TGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCTCAGACCAGCTACGGATCGTCGCCTTGGTGCGCCTTTACCACACCAACT : 1131
DH12: TGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCATCCTCTCAGACCAGCTACGGATCGTCGCCTTGGTGCGCCTTTACCACACCAACT : 1131
DH5 : TGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCTCAGACCAGCTACGGATCGTCGCCTTGGTGCGCCTTTACCACACCAACT : 1130
UV5 : TGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCTCAGACCAGCTACGGATCGTCGCCTTGGTGCGCCTTTACCACACCAACT : 1131
UV6 : TGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCTCAGACCAGCTACGGATCGTCGCCTTGGTGCGCCTTTACCACACCAACT : 1131
      TGGGCCGTGTCTCAGTCCCAGTGTGGCtGATCATCCTCTCAGACCAGCTACGGATCGTCGCCTTGGTGCGCCTTTACCACACCAACT

          1140         *       1160         *       1180         *       1200         *         12
AD27: AGCTAATCCGACGCGGGTTCATCCAATGGCGATAAATCTTTCCCCTTGCGGGCTCATACGGTATTATTTCCAGTTTCCCAGAGCTAT : 1218
DH2 : AGCTAATCCGACGCGGGTTCATCCAATGGCGATAAATCTTTCCCCTTGCGGGCTCATACGGTATTATTTCCAGTTTCCCAGAGCTAT : 1218
DH12: AGCTAATCCGACGCGGGTTCATCCAATGGCGATAAATCTTTCCCCTTGCGGGCTCATACGGTATTATTTCCAGTTTCCCAGAGCTAT : 1218
DH5 : AGCTAATCCGACGCGGGTTCATCCAATGGCGATAAATCTTTCCCCTTGCGGGCTCATACGGTATTATTTCCAGTTTCCCAGAGCTAT : 1217
UV5 : AGCTAATCCGACGCGGGTTCATCCAATGGCGATAAATCTTTCCCCTTGCGGGCTCATACGGTATTATTTCCAGTTTCCCAGAGCTAT : 1218
UV6 : AGCTAATCCGACGCGGGTTCATCCAATGGCGATAAATCTTTCCCCTTGCGGGCTCATACGGTATTATTTCCAGTTTCCCAGAGCTAT : 1218
      AGCTAATCCGACGCGGGTTCATCCAATGGCGATAAATCTTTCCCCTTGCGGGCTCATACGGTATTATTTCCAGTTTCCCAGAGCTAT

      20          *       1240         *       1260         *       1280         *       1300
AD27: TCCGTACCATTGGGCAGATCCCCACGTGTTACTCACCCGTCTGCCACTCCCCTTGCGGGGCGTTCGACTTGCATGTGTTAGGCCTG : 1304
DH2 : TCCGTACCATTGGGCAGATCCCCACGTGTTACTCACCCGTCTGCCACTCCCCTTGCGGGGCGTTCGACTTGCATGTGTTAGGCCTG : 1304
DH12: TCCGTACCATTGGGCAGATCCCCACGTGTTACTCACCCGTCTGCCACTCCCCTTGCGGGGCGTTCGACTTGCATGTGTTAGGCCTG : 1304
DH5 : TCCGTACCATTGGGCAGATCCCCACGTGTTACTCACCCGTCTGCCACTCCCCTTGCGGGGCGTTCGACTTGCATGTGTTAGGCCTG : 1303
UV5 : TCCGTACCATTGGGCAGATCCCCACGTGTTACTCACCCGTCTGCCACTCCCCTTGCGGGGCGTTCGACTTGCATGTGTTAGGCCTG : 1304
UV6 : TCCGTACCATTGGGCAGATCCCCACGTGTTACTCACCCGTCTGCCACTCCCCTTGCGGGGCGTTCGACTTGCATGTGTTAGGCCTG : 1304
      TCCGTACCATTGGGCAGATCCCCACGTGTTACTCACCCGTCTGCCACTCCCCTTGCGGGGCGTTCGACTTGCATGTGTTAGGCCTG
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Table 7.1:   Accession numbers of partial 16S rDNA gene sequences of new isolates

Isolate	Accession Number
DH2	FJ572205
DH5	FJ572206
DH12	FJ572207
UV5	FJ572208
UV6	FJ572209

Appendix B: Alignment of *dhlA* gene sequences of five newly isolated dehalogenase producers with the *dhlA* genes of *Xanthobacter autotrophicus* GJ10 and *X. flavus* UE15. Conserved regions are darkly shaded while non-conserved regions appear lighter.

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             1         *        20         *        40         *
XautoGJ10 : ATGATAAATGCAATTCGCACCCCGGACCAACGCTTCAGCAATCTCGATCAGTATCCGTT :  59
XflavUE15 : ATGATAAATGCAATTCGCACCCCGGACCAACGCTTCAGCAATCTCGATCAGTATCCGTT :  59
AaDH2     : ATGATAAATGCAATTCGCACCCCGGACCAACGCTTCAGCAATCTCGATCAGTATCCGTT :  59
AaDH5     : ATGATAAATGCAATTCGCACCCCGGACCAACGCTTCAGCAATCTCGATCAGTATCCGTT :  59
AaDH12    : ATGATAAATGCAATTCGCACCCCGGACCAACGCTTCAGCAATCTCGATCAGTATCCGTT :  59
AaUV5     : ATGATAAATGCAATTCGCACCCCGGACCAACGCTTCAGCAATCTCGATCAGTATCCGTT :  59
AaUV6     : ATGATAAATGCAATTCGCACCCCGGACCAACGCTTCAGCAATCTCGATCAGTATCCGTT :  59
            ATGATAAATGCAATTCGCACCCCGGACCAACGCTTCAGCAATCTCGATCAGTATCCGTT

             60        *        80         *       100         *
XautoGJ10 : CAGCCCCAACTACCTGGACGACCTCCCCGGCTACCCGGGATTGCGGGCACACTACCTCG : 118
XflavUE15 : CAGCCCCAACTACCTGGACGACCTCCCCGGCTACCCGGGATTGCGGGCACACTACCTCG : 118
AaDH2     : CAGCCCCAACTACCTGGACGACCTCCCCGGCTACCCGGGATTGCGGGCACACTACCTCG : 118
AaDH5     : CAGCCCCAACTACCTGGACGACCTCCCCGGCTACCCGGGATTGCGGGCACACTACCTCG : 118
AaDH12    : CAGCCCCAACTACCTGGACGACCTCCCCGGCTACCCGGGATTGCGGGCACACTACCTCG : 118
AaUV5     : CAGCCCCAACTACCTGGACGACCTCCCCGGCTACCCGGGATTGCGGGCACACTACCTCG : 118
AaUV6     : CAGCCCCAACTACCTGGACGACCTCCCCGGCTACCCGGGATTGCGGGCACACTACCTCG : 118
            CAGCCCCAACTACCTGGACGACCTCCCCGGCTACCCGGGATTGCGGGCACACTACCTCG

             120       *       140         *       160         *
XautoGJ10 : ACGAGGGCAATTCTGACGCTGAAGACGTTTTTCTCTGCCTTCATGGCGAGCCCACCTGG : 177
XflavUE15 : ACGAGGGCAATTCTGACGCTGAAGACGTTTTTCTCTGCCTTCATGGCGAGCCCACCTGG : 177
AaDH2     : ACGAGGGCAATTCTGACGCTGAAGACGTTTTTCTCTGCCTTCATGGCGAGCCCACCTGG : 177
AaDH5     : ACGAGGGCAATTCTGACGCTGAAGACGTTTTTCTCTGCCTTCATGGCGAGCCCACCTGG : 177
AaDH12    : ACGAGGGCAATTCTGACGCTGAAGACGTTTTTCTCTGCCTTCATGGCGAGCCCACCTGG : 177
AaUV5     : ACGAGGGCAATTCTGACGCTGAAGACGTTTTTCTCTGCCTTCATGGCGAGCCCACCTGG : 177
AaUV6     : ACGAGGGCAATTCTGACGCTGAAGACGTTTTTCTCTGCCTTCATGGCGAGCCCACCTGG : 177
            ACGAGGGCAATTCTGACGCTGAAGACGTTTTTCTCTGCCTTCATGGCGAGCCCACCTGG

             180       *       200         *       220         *
XautoGJ10 : AGTTACCTGTATCGCAAGATGATCCCGGTATTTGCTGAATCAGGCGCACGAGTTATTGC : 236
XflavUE15 : AGTTACCTGTATCGCAAGATGATCCCGGTATTTGCTGAATCAGGCGCACGAGTTATTGC : 236
AaDH2     : AGTTACCTGTATCGCAAGATGATCCCGGTATTTGCTGAATCAGGCGCACGAGTTATTGC : 236
AaDH5     : AGTTACCTGTATCGCAAGATGATCCCGGTATTTGCTGAATCAGGCGCACGAGTTATTGC : 236
AaDH12    : AGTTACCTGTATCGCAAGATGATCCCGGTATTTGCTGAATCAGGCGCACGAGTTATTGC : 236
AaUV5     : AGTTACCTGTATCGCAAGATGATCCCGGTATTTGCTGAATCAGGCGCACGAGTTATTGC : 236
AaUV6     : AGTTACCTGTATCGCAAGATGATCCCGGTATTTGCTGAATCAGGCGCACGAGTTATTGC : 236
            AGTTACCTGTATCGCAAGATGATCCCGGTATTTGCTGAATCAGGCGCACGAGTTATTGC

             240       *       260         *       280         *
XautoGJ10 : GCCAGACTTTTTTGGATTCGGAAAATCCGACAAGCCAGTAGACGAAGAAGACTACACCT : 295
XflavUE15 : GCCAGACTTTTTTGGATTCGGAAAATCCGACAAGCCAGTAGACGAAGAAGACTACACCT : 295
AaDH2     : GCCAGACTTTTTTGGATTCGGAAAATCCGACAAGCCAGTAGACGAAGAAGACTACACCT : 295
AaDH5     : GCCAGACTTTTTTGGATTCGGAAAATCCGACAAGCCAGTAGACGAAGAAGACTACACCT : 295
AaDH12    : GCCAGACTTTTTTGGATTCGGAAAATCCGACAAGCCAGTAGACGAAGAAGACTACACCT : 295
AaUV5     : GCCAGACTTTTTTGGATTCGGAAAATCCGACAAGCCAGTAGACGAAGAAGACTACACCT : 295
AaUV6     : GCCAGACTTTTTTGGATTCGGAAAATCCGACAAGCCAGTAGACGAAGAAGACTACACCT : 295
            GCCAGACTTTTTTGGATTCGGAAAATCCGACAAGCCAGTAGACGAAGAAGACTACACCT

             300       *       320         *       340         *
XautoGJ10 : TCGAATTTCACCGCAACTTCCTGCTTGCACTAATCGAACGGCTTGACTTGCGCAACATT : 354
XflavUE15 : TCGAATTTCACCGCAACTTCCTGCTTGCACTAATCGAACGGCTTGACTTGCGCAACATT : 354
AaDH2     : TCGAATTTCACCGCAACTTCCTGCTTGCACTAATCGAACGGCTTGACTTGCGCAACATT : 354
AaDH5     : TCGAATTTCACCGCAACTTCCTGCTTGCACTAATCGAACGGCTTGACTTGCGCAACATT : 354
AaDH12    : TCGAATTTCACCGCAACTTCCTGCTTGCACTAATCGAACGGCTTGACTTGCGCAACATT : 354
AaUV5     : TCGAATTTCACCGCAACTTCCTGCTTGCACTAATCGAACGGCTTGACTTGCGCAACATT : 354
AaUV6     : TCGAATTTCACCGCAACTTCCTGCTTGCACTAATCGAACGGCTTGACTTGCGCAACATT : 354
            TCGAATTTCACCGCAACTTCCTGCTTGCACTAATCGAACGGCTTGACTTGCGCAACATT
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                360        *        380        *        400        *
XautoGJ10 : ACGCTGGTCGTTCAGGACTGGGGCGGATTTTTGGGGCTGACCTTACCGATGGCCGACCC : 413
XflavUE15 : ACGCTGGTCGTTCAGGACTGGGGCGGATTTTTGGGGCTGACCTTACCGATGGCCGACCC : 413
AaDH2     : ACGCTGGTCGTTCAGGACTGGGGCGGATTTTTGGGGCTGACCTTACCGATGGCCGACCC : 413
AaDH5     : ACGCTGGTCGTTCAGGACTGGGGCGGATTTTTGGGGCTGACCTTACCGATGGCCGACCC : 413
AaDH12    : ACGCTGGTCGTTCAGGACTGGGGCGGATTTTTGGGGCTGACCTTACCGATGGCCGACCC : 413
AaUV5     : ACGCTGGTCGTTCAGGACTGGGGCGGATTTTTGGGGCTGACCTTACCGATGGCCGACCC : 413
AaUV6     : ACGCTGGTCGTTCAGGACTGGGGCGGATTTTTGGGGCTGACCTTACCGATGGCCGACCC : 413
            ACGCTGGTCGTTCAGGACTGGGGCGGATTTTTGGGGCTGACCTTACCGATGGCCGACCC

                420        *        440        *        460        *
XautoGJ10 : TTCCCGCTTCAAGCGCCTGATCATCATGAACGCCTGCTTGATGACCGACCCGGTCACCC : 472
XflavUE15 : TTCCCGCTTCAAGCGCCTGATCATCATGAACGCCTGCTTGATGACCGACCCGGTCACCC : 472
AaDH2     : TTCCCGCTTCAAGCGCCTGATCATCATGAACGCCTGCTTGATGACCGACCCGGTCACCC : 472
AaDH5     : TTCCCGCTTCAAGCGCCTGATCATCATGAACGCCTGCTTGATGACCGACCCGGTCACCC : 472
AaDH12    : TTCCCGCTTCAAGCGCCTGATCATCATGAACGCCTGCTTGATGACCGACCCGGTCACCC : 472
AaUV5     : TTCCCGCTTCAAGCGCCTGATCATCATGAACGCCTGCTTGATGACCGACCCGGTCACCC : 472
AaUV6     : TTCCCGCTTCAAGCGCCTGATCATCATGAACGCCTGCTTGATGACCGACCCGGTCACCC : 472
            TTCCCGCTTCAAGCGCCTGATCATCATGAACGCCTGCTTGATGACCGACCCGGTCACCC

                480        *        500        *        520        *
XautoGJ10 : AGCCTGCGTTTAGCGCCTTTGTCACCCAGCCTGCGGATGGCTTTACCGCCTGGAAATAC : 531
XflavUE15 : AGCCTGCGTTTAGCGCCTTTGTCACCCAGCCTGCGGATGGCTTTACCGCCTGGAAATAC : 531
AaDH2     : AGCCTGCGTTTAGCGCCTTTGTCACCCAGCCTGCGGATGGCTTTACCGCCTGGAAATAC : 531
AaDH5     : AGCCTGCGTTTAGCGCCTTTGTCACCCAGCCTGCGGATGGCTTTACCGCCTGGAAATAC : 531
AaDH12    : AGCCTGCGTTTAGCGCCTTTGTCACCCAGCCTGCGGATGGCTTTACCGCCTGGAAATAC : 531
AaUV5     : AGCCTGCGTTTAGCGCCTTTGTCACCCAGCCTGCGGATGGCTTTACCGCCTGGAAATAC : 531
AaUV6     : AGCCTGCGTTTAGCGCCTTTGTCACCCAGCCTGCGGATGGCTTTACCGCCTGGAAATAC : 531
            AGCCTGCGTTTAGCGCCTTTGTCACCCAGCCTGCGGATGGCTTTACCGCCTGGAAATAC

                540        *        560        *        580        *
XautoGJ10 : GATCTGGTTACGCCATCAGACCTGCGCCTTGACCAGTTCATGAAGCGTTGGGCGCCCAC : 590
XflavUE15 : GATCTGGTTACGCCATCAGACCTGCGCCTTGACCAGTTCATGAAGCGTTGGGCGCCCAC : 590
AaDH2     : GATCTGGTTACGCCATCAGACCTGCGCCTTGACCAGTTCATGAAGCGTTGGGCGCCCAC : 590
AaDH5     : GATCTGGTTACGCCATCAGACCTGCGCCTTGACCAGTTCATGAAGCGTTGGGCGCCCAC : 590
AaDH12    : GATCTGGTTACGCCATCAGACCTGCGCCTTGACCAGTTCATGAAGCGTTGGGCGCCCAC : 590
AaUV5     : GATCTGGTTACGCCATCAGACCTGCGCCTTGACCAGTTCATGAAGCGTTGGGCGCCCAC : 590
AaUV6     : GATCTGGTTACGCCATCAGACCTGCGCCTTGACCAGTTCATGAAGCGTTGGGCGCCCAC : 590
            GATCTGGTTACGCCATCAGACCTGCGCCTTGACCAGTTCATGAAGCGTTGGGCGCCCAC

                600        *        620        *        640
XautoGJ10 : ACTGACCGAAGCTGAGGCCTCCGCGTATGCTGCGCCTTTCCCTGACACTTCCTATCAGG : 649
XflavUE15 : ACTGACCGAAGCTGAGGCCTCCGCGTATGCTGCGCCTTTCCCTGACACTTCCTATCAGG : 649
AaDH2     : ACTGACCGAAGCTGAGGCCTCCGCGTATGCTGCGCCTTTCCCTGACACTTCCTATCAGG : 649
AaDH5     : ACTGACCGAAGCTGAGGCCTCCGCGTATGCTGCGCCTTTCCCTGACACTTCCTATCAGG : 649
AaDH12    : ACTGACCGAAGCTGAGGCCTCCGCGTATGCTGCGCCTTTCCCTGACACTTCCTATCAGG : 649
AaUV5     : ACTGACCGAAGCTGAGGCCTCCGCGTATGCTGCGCCTTTCCCTGACACTTCCTATCAGG : 649
AaUV6     : ACTGACCGAAGCTGAGGCCTCCGCGTATGCTGCGCCTTTCCCTGACACTTCCTATCAGG : 649
            ACTGACCGAAGCTGAGGCCTCCGCGTATGCTGCGCCTTTCCCTGACACTTCCTATCAGG

            *        660        *        680        *        700
XautoGJ10 : CTGGTGTACGCAAGTTTCCCAAGATGGTCGCGCAACGCGACCAGGCCTGCATCGACATT : 708
XflavUE15 : CTGGTGTACGCAAGTTTCCCAAGATGGTCGCGCAACGCGACCAGGCCTGCATCGACATT : 708
AaDH2     : CTGGTGTACGCAAGTTTCCCAAGATGGTCGCGCAACGCGACCAGGCCTGCATCGACATT : 708
AaDH5     : CTGGTGTACGCAAGTTTCCCAAGATGGTCGCGCAACGCGACCAGGCCTGCATCGACATT : 708
AaDH12    : CTGGTGTACGCAAGTTTCCCAAGATGGTCGCGCAACGCGACCAGGCCTGCATCGACATT : 708
AaUV5     : CTGGTGTACGCAAGTTTCCCAAGATGGTCGCGCAACGCGACCAGGCCTGCATCGACATT : 708
AaUV6     : CTGGTGTACGCAAGTTTCCCAAGATGGTCGCGCAACGCGACCAGGCCTGCATCGACATT : 708
            CTGGTGTACGCAAGTTTCCCAAGATGGTCGCGCAACGCGACCAGGCCTGCATCGACATT

            *        720        *        740        *        760
XautoGJ10 : TCAACCGAAGCGATTTCGTTCTGGCAGAACGACTGGAATGGCCAGACCTTCATGGCCAT : 767
XflavUE15 : TCAACCGAAGCGATTTCGTTCTGGCAGAACGACTGGAATGGCCAGACCTTCATGGCCAT : 767
AaDH2     : TCAACCGAAGCGATTTCGTTCTGGCAGAACGACTGGAATGGCCAGACCTTCATGGCCAT : 767
AaDH5     : TCAACCGAAGCGATTTCGTTCTGGCAGAACGACTGGAATGGCCAGACCTTCATGGCCAT : 767
AaDH12    : TCAACCGAAGCGATTTCGTTCTGGCAGAACGACTGGAATGGCCAGACCTTCATGGCCAT : 767
AaUV5     : TCAACCGAAGCGATTTCGTTCTGGCAGAACGACTGGAATGGCCAGACCTTCATGGCCAT : 767
AaUV6     : TCAACCGAAGCGATTTCGTTCTGGCAGAACGACTGGAATGGCCAGACCTTCATGGCCAT : 767
            TCAACCGAAGCGATTTCGTTCTGGCAGAACGACTGGAATGGCCAGACCTTCATGGCCAT

            *        780        *        800        *        820
XautoGJ10 : TGGCATGAAAGACAAATTGCTGGGACCGGACGTCATGTATCCTATGAAGGCGCTCATTA : 826
XflavUE15 : TGGCATGAAAGACAAATTGCTGGGACCGGACGTCATGTATCCTATGAAGGCGCTCATTA : 826
AaDH2     : TGGCATGAAAGACAAATTGCTGGGACCGGACGTCATGTATCCTATGAAGGCGCTCATTA : 826
AaDH5     : TGGCATGAAAGACAAATTGCTGGGACCGGACGTCATGTATCCTATGAAGGCGCTCATTA : 826
AaDH12    : TGGCATGAAAGACAAATTGCTGGGACCGGACGTCATGTATCCTATGAAGGCGCTCATTA : 826
AaUV5     : TGGCATGAAAGACAAATTGCTGGGACCGGACGTCATGTATCCTATGAAGGCGCTCATTA : 826
AaUV6     : TGGCATGAAAGACAAATTGCTGGGACCGGACGTCATGTATCCTATGAAGGCGCTCATTA : 826
            TGGCATGAAAGACAAATTGCTGGGACCGGACGTCATGTATCCTATGAAGGCGCTCATTA
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205

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                    *         840       *         860       *         880
XautoGJ10 : ATGGCTGCCCGGAACCCCTCGAAATAGCGGACGCTGGCCATTTCGTACAGGAGTTTGGC : 885
XflavUE15 : ATGGCTGCCCGGAACCCCTCGAAATAGCGGACGCTGGCCATTTCGTACAGGAGTTTGGC : 885
AaDH2     : ATGGCTGCCCGGAACCCCTCGAAATAGCGGACGCTGGCCATTTCGTACAGGAGTTTGGC : 885
AaDH5     : ATGGCTGCCCGGAACCCCTCGAAATAGCGGACGCTGGCCATTTCGTACAGGAGTTTGGC : 885
AaDH12    : ATGGCTGCCCGGAACCCCTCGAAATAGCGGACGCTGGCCATTTCGTACAGGAGTTTGGC : 885
AaUV5     : ATGGCTGCCCGGAACCCCTCGAAATAGCGGACGCTGGCCATTTCGTACAGGAGTTTGGC : 885
AaUV6     : ATGGCTGCCCGGAACCCCTCGAAATAGCGGACGCTGGCCATTTCGTACAGGAGTTTGGC : 885
            ATGGCTGCCCGGAACCCCTCGAAATAGCGGACGCTGGCCATTTCGTACAGGAGTTTGGC

                    *         900       *         920       *
XautoGJ10 : GAGCAAGTGGCTCGCGAGGCCCTGAAACACTTTGCCGAGACAGAATAG : 933
XflavUE15 : GAGCAAGTGGCTCGCGAGGCCCTGAAACACTTTGCCGAGACAGAATAG : 933
AaDH2     : GAGCAAGTGGCTCGCGAGGCCCTGAAACACTTTGCCGAGACAGAATAG : 933
AaDH5     : GAGCAAGTGGCTCGCGAGGCCCTGAAACACTTTGCCGAGACAGAATAG : 933
AaDH12    : GAGCAAGTGGCTCGCGAGGCCCTGAAACACTTTGCCGAGACAGAATAG : 933
AaUV5     : GAGCAAGTGGCTCGCGAGGCCCTGAAACACTTTGCCGAGACAGAATAG : 933
AaUV6     : GAGCAAGTGGCTCGCGAGGCCCTGAAACACTTTGCCGAGACAGAATAG : 933
            GAGCAAGTGGCTCGCGAGGCCCTGAAACACTTTGCCGAGACAGAATAG
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Table 7.2: Accession numbers of *dhlA* gene sequences of new isolates

Isolate	Accession Number
DH2	FJ573162
DH5	FJ573163
DH12	FJ573164
UV5	FJ573165
UV6	FJ573166