ANALYSIS OF *RHIZOBIUM SP.*
DEHALOGENASES AND THEIR REGULATION

This thesis is submitted for the degree of
Doctor of Philosophy
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By

Fahrul Zaman Huyop M.Sc. (Bristol)

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ABSTRACT

Analysis of Rhizobium sp. Dehalogenases and their Regulation

Fahrul Zaman Huyop

The genus of the organism was confirmed as Rhizobium by partial sequencing of its 16S rRNA gene. The Rhizobium sp. DehL, DehE and DehD were produced by heterologous expression of the cloned genes in Escherichia coli K-12 and the proteins purified. All three dehalogenases were further characterised by kinetic analysis. The Km, Kcat and the Specificity Constants of these enzymes were determined. Rhizobium sp. was able to grow at 0.2mM 2,2DCP, which was 100x lower than the concentration of the substrate routinely used. Apparently, no new dehalogenases are required to allow growth on this low concentration of 2,2DCP as judged by electrophoretic mobility of dehalogenase proteins on Native-PAGE analysis and protein separation by anion-exchange column chromatography. The kinetic analysis suggested that the known dehalogenases were able to act efficiently on low concentrations of haloalkanoic acids. The amount of each dehalogenase, from cells grown on low substrate concentration was different to that seen at 20mM 2,2DCP due to complex regulatory controls, which respond to the growth environment. The cloning of the putative Rhizobium sp. regulator gene was achieved using phenotypic co-selection and the deduced amino acid sequence was compared to the databases. The putative regulatory sequence of Rhizobium sp. was highly homologous to the DehR1 of Pseudomonas putida PP3 with a sequence identity of 72% and the DhlR of Xanthobacter autotrophicus GJ10 with 48% identity. The regulatory gene was cloned into a high expression vector but the protein produced was found in inclusion bodies and presumably not active. The regulator gene was then characterised using an in vivo system by ligating the dehD and dehL structural genes into the same plasmid where the regulator gene itself was controlled by the vector promoter system. The regulator gene product was capable of controlling the expression of both dehD and dehL in the presence of D,L2-CP (inducer), and the promoter sequence. It was not possible by such a procedure to detect the regulation of expression of dehE, possibly due to the presumed absence of its putative promoter sequence in the construct made.
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<table>
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<tr>
<td>Amp</td>
<td>ampicillin</td>
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<tr>
<td>A</td>
<td>absorbance</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cetyltrimethyl ammonium bromide</td>
</tr>
<tr>
<td>DCA</td>
<td>dichloroacetic acid</td>
</tr>
<tr>
<td>DBA</td>
<td>dibromoacetic acid</td>
</tr>
<tr>
<td>DehE</td>
<td>dehalogenase II</td>
</tr>
<tr>
<td>DehD</td>
<td>dehalogenase III</td>
</tr>
<tr>
<td>DehL</td>
<td>dehalogenase I</td>
</tr>
<tr>
<td>2,2DCP</td>
<td>2,2-dichloropropionic acid</td>
</tr>
<tr>
<td>D,L2,3-DCP</td>
<td>D,L-2,3-dichloropropionic acid</td>
</tr>
<tr>
<td>D,L2-CP</td>
<td>D,L-2-chloropropionic acid</td>
</tr>
<tr>
<td>D,L2-BP</td>
<td>D,L-2-bromopropionic acid</td>
</tr>
<tr>
<td>D-2CP</td>
<td>D-2-chloropropionic acid</td>
</tr>
<tr>
<td>L-2CP</td>
<td>L-2-chloropropionic acid</td>
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<tr>
<td>D-2BP</td>
<td>D-2-bromopropionic acid</td>
</tr>
<tr>
<td>L-2BP</td>
<td>L-2-bromopropionic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid (Na₂ salt)</td>
</tr>
<tr>
<td>HAA</td>
<td>halogenated alkanoic acid</td>
</tr>
<tr>
<td>IAA</td>
<td>Isoamyl alcohol</td>
</tr>
<tr>
<td>IMS</td>
<td>industrial methylated spirit</td>
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<tr>
<td>IPTG</td>
<td>Isopropylthio-β-D-galactoside</td>
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<tr>
<td>kb</td>
<td>kilobase pair</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Daltons</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Broth</td>
</tr>
<tr>
<td>MCA</td>
<td>Monochloroacetic acid</td>
</tr>
<tr>
<td>MBA</td>
<td>Monobromoacetic acid</td>
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<tr>
<td>MOPS</td>
<td>3-[N-morpholino]propanesulphonic acid</td>
</tr>
<tr>
<td>NTA</td>
<td>Nitrilo Tri-acetic Acid</td>
</tr>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecylsulphate</td>
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<tr>
<td>TAE</td>
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<tr>
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<tr>
<td>TE</td>
<td>Tris EDTA</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylenediamine</td>
</tr>
<tr>
<td>Tris</td>
<td>Trihydroxymethylaminomethane</td>
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<td>UV</td>
<td>ultra violet</td>
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AUTHOR DECLARATIONS

This thesis, submitted for the degree of Doctor of Philosophy entitled: Analysis of Rhizobium sp. Dehalogenases and their Regulation, is based upon work conducted by the author in the Department of Biochemistry at the University of Leicester between February 1998 and March 2001.

All of the work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

None of this work has been submitted for another degree in this or any other University.

Signed: Date:
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CHAPTER 7

Figure 7.1. pSC1

Figure 7.2. pSC530
1.1 The existence of naturally halogenated organic compounds

Natural organic compounds containing carbon-halogen covalent bonds are found widely throughout the biosphere in plants and animals (Fowden, 1968). According to Wackett (1991), over 700 such compounds have been isolated from both prokaryotes and eukaryotes. The major group is chlorinated compounds followed by brominated compounds with fluorinated and iodinated compounds few in number (Siuda and DeBernadis, 1973). The role of many of these compounds is suggested to inhibit the growth of competing species (for example production of antibiotics, tetracycline and chloramphenicol). It is the release of man-made compounds, however, that has raised awareness of environmental issues relating to halogenated compounds.

Marais (1944) showed that fluoroacetate was the toxic principle in the South African plant *Dichapetalum cymnosum*. Fluoroacetate has also been discovered in other higher plants such as *Palicourea marcgravii* (Oliveira, 1963), *Acacia georginae* (Oelrichs and McEwan, 1962), *Gastrolubium species* and *Oxylobium carium* in the leaves (McEwan 1964; Aplin, 1967). The toxicity of fluoroacetate is due to its *in vivo* conversion to fluoroacetyl co-enzyme A and the subsequent ‘lethal synthesis’ of fluorocitrate, a potent inhibitor of the Krebs cycle enzyme aconitase (Morrison and Peters, 1954). Other chlorinated compounds such as chlorinated naphthoquinones, sesquiterpenes and indoles have been isolated from plants. Derivatives of 4-chloroindole isolated from the immature seeds of *Pisum sativum* (Marumo and Hattori, 1970) are known to be plant auxins. However, the natural function of many other halogenated organic compounds synthesised by plants remains unclear.

Higher animals synthesise very few halogenated compounds. However, the thyroid gland of vertebrates contains large amounts of iodo-substituted tyrosines. These compounds are pre-cursors of the thyroid hormones for example 3,3',5,5'-tetraiodothyronine, thyroxine and 3,3',5-triiodothyronine which are largely responsible for controlling animal growth and development (Grodsky, 1979).

Iodinated tyrosines have also been detected in marine organisms such as seaweeds and sponges (Fowden, 1968). Most of the naturally occurring brominated compounds are found in marine organisms and are used as dyes. For example sea molluscs, *Murex brandaris*, produced Tyrian purple from 6,6'-Dibromoindigotin (Figure 1.1). The
colour of red algae and red sponges is largely due to complex brominated pigments. An unusual bromine-rich antibiotic containing bromo analogs of pyrrolnitrins [2,3,4-tribromo-5 (1'-hydroxy, 2'4'-dibromophenyl) pyrrole] isolated from a marine Pseudomonad (Burkholder, et al., 1966) was active against Gram-positive bacteria.

The major producers of halogenated organic compounds are lichens, fungi and bacteria. Most of the halo metabolites synthesised contain chlorine as the halogen. Many other chlorinated metabolites possess a wide range of biological activities. Some of them exhibit antimicrobial activity and a few also show an anti-tumour activity. Notable examples are chlorotetracycline, from Streptomyces aureofaciens, chloramphenicol from Streptomyces venezuelae and griseofulvin from Penicillium griseofulvum (Figure 1.1). Brominated phenols and pyrroles also showed antimicrobial activity but apparently most are too toxic or insufficiently potent to be used effectively. Unusual fluorinated compounds such as nucleodin were isolated from Streptomyces clavus (Tobie, 1957; Thomas et al., 1956) and reported to be antitrypanosomal antibiotics. Removal of the halogen from an antibiotic does not always result in loss of biological activity. Tetracycline is as effective an antimicrobial as chlorotetracycline and removal of the dichloroacetyl group from chloramphenicol results in the no loss of antibiotic properties (Vazquez, 1966).

Some fungal halo metabolites have proved to be potent toxins. Foodstuffs contaminated with either Aspergillus or Penicillium sp. (Steyn, 1971) were found to contain Ochratoxin A, a chlorinated dihydroisocoumarin. Higher fungi such as Basidiomycetes have a widespread capacity for organohalogen biosynthesis as reported recently (Ed de Jong, 1997). Sulphur tuft (Hypholoma fasciculare) was among the highest organohalogen producers. These metabolites are found to have physiological roles as antibiotics and as metabolites involved in lignin degradation. Extensive reviews on naturally occurring halogenated compounds have been published by Fowden (1968), Suida and Debernadis (1973) and Wackett (1991).

In fact there are such a large number of halogenated organic compounds available in the environment that microorganisms might have evolved their metabolic pathways to metabolise such halogenated compounds.
Figure 1.1 Halometabolites produced by eukaryotic organisms (adapted from van Pee, 1996)
1.2 Synthetic halogenated organic compounds

There has been wide use of synthetic halogenated compounds by man for a variety purposes for example as herbicides, insecticides, fungicides, drugs, aerosols, propellants, plastics, industrial solvents and refrigerants. Some of these materials are rich in toxic substances and persist in the environment. Their presence has become a cause for concern, as they are a significant problem as pollutants (Alexander, 1999).

Pesticides and herbicides have been of great help to crop production. They protect crop plants from injuries and damage caused by harmful insects and mites, infectious fungi and bacteria and invasive weeds (Ashton and Crafts, 1973). Halogenated alkanoic acids are major components in some herbicides for example trichloroacetic acid (TCA) or 2,2-dichloropropionic acid (2,2DCP; Dalapon) which have been used as herbicides since the 1950’s. TCA (McCall and Zahnley, 1949) and the sodium salt of 2,2DCP (Dow Chemical, 1953) were found to be selectively herbicidal. Dalapon was used as foliar treatment to control perennial grasses in certain crops and on non-crop land (Leasure, 1964). The phytotoxic symptoms of the chlorinated aliphatic acid herbicides are growth inhibition, leave chlorosis and formative effects especially at the shoot apex (Ashton and Crafts, 1973). Dalapon is absorbed by both foliage and roots and translocated within the plant largely as the unchanged chemical (Foy, 1960). TCA is absorbed primarily by the roots but neither this substance nor Dalapon appear to undergo significant degradation in a plant. All chlorinated aliphatic acid herbicides exhibit very similar phytotoxic effects. Foy (1969), reviewed the degradation of TCA and 2,2DCP in plants, animals and soils. In general they are quite stable in plants and animals but degraded rapidly in soil. Kearney et al.,(1965), suggested that 2,2DCP and TCA can only exist in the unionised form at very low pH values and at normal physiological pH would be the anionic form. At higher concentrations both Dalapon and TCA cause necrosis and severe contact injury. This latter has been attributed to the protein precipitant properties of these chemicals (Redemann, 1954). The precise mechanism of action of Dalapon and TCA at the cellular level in higher plants is still unknown (Foy, 1975).
Active ingredients of herbicides such as chlorophenoxy acids, 2,4-dichlorophenoxyacetic acid (2,4D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) have been used widely since the 1940's (Loos, 1975). Most of these herbicides are easily degraded by soil organisms to simple metabolic products. However, there has been controversy over the continued use of 2,4,5-T as a herbicide following reports of its teratogenic effects upon certain mammals (Kearney and Kaufman, 1972; Loos, 1975). Most of the substituted urea herbicides currently in use contain halogens attached to an aromatic ring. In most cases the halogen is chlorine, for example Diuron [3-(3,4-dichlorophenyl)-1,1dimethylurea] and Monuron [3-(p-chlorophenyl)-1,1-dimethyl urea] but bromine, fluorine and sometimes two different halogens attached to the same aromatic nucleus occur in these herbicides like Chlorobromuron[3-(4-bromo-3-chlorophenyl)-1-methoxy1methylurea]. These are all selective herbicides, where their main mode of action is to inhibit the photosynthesis process in plants. Soil microorganisms are known to degrade these herbicides to aniline or substituted aniline molecules, but further metabolism is unclear (Kearney and Kaufman, 1972; Geissbuhler et al., 1975; Cripps and Roberts, 1978).

A large number disinfectants in current household use contain halogenated organic compounds as their active ingredients such as in 'Dettol' Chloroxylenol (Block, 1977); Triclosan 5-chloro-2(2,4-dichlorophenoxy) phenol used as disinfectant and textile preservative (Savage, 1971) and in tooth-paste.

Polychlorobiphenyl (PCB) was first manufactured in 1929 and the production peaked in the late 1950s and early 1970s but production decreased after discovery of the wide spread environmental contamination they were causing. This material was widely used in electrical equipment as they were stable and good insulators. PCBs were also used in hydraulic equipment in factories. As reported earlier PCBs can accumulate in the food chain and cause a variety of ill effects in lab animals from liver damage to cancer (Kaiser, 2000). Developed countries banned the use of PCBs decades ago. But PCBs can still be found in minute levels in the body fat of some people. These low levels of PCBs may affect development in young children and also can weaken the infant’s immune system. Previous work also suggested that dioxins, chemicals related to PCBs, which are still produced as by-products of incineration and industrial bleaching, suppress the immune system.
Release of volatile substances is harmful to humans, animals and the biosphere in general. Gaseous chlorofluorohydrocarbons (CFC) and related substances utilised as propellants are subject to photodecomposition by ultra-violet radiation and their effect on the ozone layer of the atmosphere has become a matter for concern (Molina and Rowland, 1974).

The extensive use of halogenated compounds in agriculture and industry has led to soil pollution in many areas (Alexander, 1965). Halogenated pesticides or herbicides may be destroyed in soil by several mechanisms either biologically or non-biologically. Adsorption and inactivation by clay and organic colloidal materials or other non-biological degradation may contribute significantly to the environmental decontamination (Holly, 1964). Volatilisation or photochemical degradation can also contribute to the removal of certain chemicals from a particular ecosystem and leaching may occasionally be responsible for the removal of pesticides from surface zones of soil (Holly, 1964). However, microbiological activity is the principle means for transforming the xenobiotic substances into harmless products (Slater and Godwin, 1980).

1.3 Properties of halogenated aliphatic acids

The haloorganic compounds degraded by microorganisms are classified into three groups:

1. Haloaliphatic
2. Haloaromatic
3. Haloheterocyclic

The aliphatic acids, in which one or more aliphatic chain hydrogen atom has been replaced with a halogen are amongst the simplest organic compounds used as herbicides. In short-chain halogenated saturated alkanoic acids, the electronegative halogen causes a high degree of ionisation at the carboxyl group and makes these derivatives stronger acids than the parent non-halogenated acid. The more electronegative the halogen substituent the greater will be its inductive effect. This can be observed by the value of pKa of the monohalogenated acetic acids which are 2.57, 2.87, 2.89 and 3.17 for monofluoro-, monochloro-, monobromo- and moniodo-acetic acid respectively (Zabicky and Ehrlich- Rogozinski, 1973).
Fluorine, being the most electronegative of the halogens, forms the strongest acid in the monohaloacetic group. Chlorine, bromine and iodine are less electronegative than fluorine and form weaker acids. When compared to the parent acetic acid, pKa 4.76, all the four monohalogenated derivatives are stronger acids. Increasing the number of halogen substituents in an aliphatic chain leads to an increase in the nett inductive effect and to the formation of progressively stronger acids. For example, trichloroacetic acid with a pKa value of 0.15, is a stronger acid than both dichloroacetic and monochloroacetic acids with pKas of 1.29 and 2.87, respectively (Zabicky and Ehrlich-Rogozinski, 1973).

The position of the halogen substituents in the aliphatic chains may greatly influence the degree of ionisation of the carboxyl group. The nearer the halogen to the carboxyl group the greater will be its inductive effect. Thus, β and γ substituted butyric acids are weaker than the α substituted butyric acids. The presence of conjugated double bonds between the carboxyl group and the substitution sites in longer chain aliphatic acids enhances the inductive effect of the halogen and leads to an increased acidity compared with the corresponding saturated compound.

The short-chain halogenated alkanoic acids are water soluble. Similar to the non-halogenated parent compounds their solubility in water decreases with increasing chain length. Their decomposition products depend on number, nature and position of the halogens in the aliphatic chain. But all decompose readily upon heating and slowly in aqueous solution at room temperature. Some of these compounds exhibit optical isomerism. For example, α-monochloropropionic acid and α-monochlorobutyric acid possess chiral carbon atoms. The chemical reactivities of the haloaliphatic acids may be divided into those involving the carboxyl group and those involving the substituent halogens. The carboxyl group undergoes similar reactions to that of the corresponding non-halogenated acid, salts and esters being formed. A compound with substituent halogens makes the carbon atom to which it is attached open to attack by nucleophilic reagent(s). For example, when an aqueous solution of monochloroacetic acid is boiled, nucleophilic attack by an hydroxyl ion at the α carbon atom, results in the elimination of the chlorine atom and formation of glycolic acid. Similar nucleophilic substitutions are thought to happen for the biological cleavage of the carbon halogen bond of several halogenated aliphatic acids.
In TCA and 2,2DCP (Dalapon) only the α-chlorinated aliphatic acids exhibit herbicidal properties. Compounds with chlorine in other positions do not possess phytotoxic properties unless they are also α-chlorinated. Increasing chain length progressively reduces the herbicidal activity of the α-chloro acids and 2,2-dichlorohexanoic acids. Similarly, substitution of other halogens for chlorine generally decreases herbicidal activity of these acids (Leasure, 1964).

1.4 Biodegradation of halogenated alkanoic acids

The largest group of environmental pollutants are halogenated organic compounds. Man-made chemicals can cause environmental pollution and human health problems as a result of their persistence, toxicity and transformation into hazardous metabolites. Halogenation is often implicated as a reason for persistence in nature (Neilson et al., 1985). Microbial degradation of herbicides in soil plays an important role in preventing the build up of these chemicals to toxic levels. Many species of microorganisms have been reported to degrade them. The first report of organisms able to degrade halogenated aliphatic acids was made in 1957 when the term dehalogenase was first introduced (Jensen, 1951, 1957, 1959, 1960, 1963). The first study to assay dehalogenases in cell-free systems concentrated on halogenated aliphatic acids substituted in C2 position. Degradation of halogenated compounds (halo-acetate and halopropionate) in the environment involves hydrolytic removal of the halogen substituents to allow the carbon skeleton to channel into central metabolic pathways as follows (Jensen 1960; 1963):

(i) R-Cl-COO⁻ + H₂O → ROH-COO⁻ + H⁺ + Cl⁻

The following reactions involve halogen substituent replacement by a hydroxyl group derived from water (Goldman, 1972; Stringfellow, et al., 1997). The products resulting from this general scheme of metabolism would be the corresponding hydroxy or keto acids:

(ii) CH₂Cl-COO⁻ + H₂O → CH₂OH-COO⁻ + H⁺ + Cl⁻

\textbf{MCA} \quad \text{Glycollate}

(iii) CHCl₂-COO⁻ + H₂O → CHO-COO⁻ + 2H⁺ + 2Cl⁻

\textbf{DCA} \quad \text{Glyoxylate}
(iv) \( \text{CCl}_3\text{COO}^- + 2\text{H}_2\text{O} \longrightarrow \text{COOH-COO}^- + 3\text{H}^+ + 3\text{Cl}^- \)

**TCA**

(Oxalate)

(or)

\( \text{CCl}_3\text{COO}^- + \text{H}_2\text{O} \longrightarrow 3\text{Cl}^- + \text{CO} + \text{CO}_2 + 2\text{H}^+ \)

**TCA**

(v) \( \text{CH}_3\text{-CHCl-COO}^- + \text{H}_2\text{O} \longrightarrow \text{CH}_3\text{-CHOH-COO}^- + \text{H}^+ + \text{Cl}^- \)

**D,L2-CP**

(Lactate)

(vi) \( \text{CH}_3\text{-CCl}_2\text{COO}^- + \text{H}_2\text{O} \longrightarrow \text{CH}_3\text{-CO-COO}^- + 2\text{H}^+ + 2\text{Cl}^- \)

**2,2DCP**

(Pyruvate)

In early 70's some microbes were reported to be able to grow on 3-halogenated alkanoic acids, but the biochemistry and enzymology is far from clear (Bollag and Alexander, 1971; Hughes, 1988).

The basic reasons why dehalogenation of halogenated aliphatic acids is important for some microorganisms are:

1- Dehalogenation of the compounds results in the formation of molecules which are either direct intermediates of central metabolism or can be converted to intermediary metabolites. In some cases, which may happen widely in nature, the dehalogenase activities yield products not used by the dehalogenase synthesising organisms but are used to support growth of non-dehalogenase producing micro-organisms (Slater and Bull, 1982). This is known as a co-metabolic reaction.

2- Many halogenated compounds are toxic (Peters, 1952). Dehalogenation can be a detoxification mechanism aiding microbial survival in environments rather than as mechanisms for utilising a halogenated compound as a carbon and energy source for growth.
1.4.1 Classification of dehalogenase enzymes

Many dehalogenases have been isolated and characterised according to their biochemical and genetic properties. Some microorganisms were found to contain more than one dehalogenase. But the significance of multiple dehalogenases is far from clear.

Haloalkanoic acid dehalogenase enzymes have been grouped based on their dehalogenation mechanism or on the basis of substrate specificities. Although various enzymes can be grouped together it may not indicate a close relationship between the proteins involved. It is clear that there are many distinct features between these enzymes including pH optima (Slater et al., 1979), size and subunit structure (Motosugi et al., 1982a; Allison et al., 1983; Tsang et al., 1988; Smith et al., 1990), electrophoretic mobility under non-denaturing conditions and also substrate specificities (Hardman and Slater, 1981a). Hardman and Slater (1981a;b) found that the majority of organisms capable of growth on haloalkanoic acids contained more than one dehalogenase. The presence of more than one dehalogenase within a single organism can vary with the stage and manner of growth (Hardman and Slater 1981b; Tsang et al., 1988). The majority of dehalogenases were found to be inducible and not constitutively produced. Inducers for dehalogenase are not always growth substrates (Allison et al., 1983; Berry et al., 1979; Slater et al., 1979) and regulation of expression is poorly understood.

In general the grouping of different dehalogenases are primarily based on mechanisms of enzyme reactions or by looking at other factors such as substrate specificities and amino acid sequence information. The most recent classification of dehalogenase enzymes was proposed by Slater et al., (1997) which involved 3 basic groupings:

I) Hydrolytic dehalogenases
II) Haloalcohol dehalogenases
III) Co-factor dependent dehalogenases
Hydrolytic dehalogenases were the most common enzymes and divided into two categories i) 2-haloalkanoic acid hydrolytic dehalogenases and ii) haloalkane hydrolytic dehalogenases.

i) 2-haloalkanoic acid hydrolytic dehalogenases

Table 1.1. Class ID: D-isomer specific – inverts substrate product configuration

<table>
<thead>
<tr>
<th>Organisms – Dehalogenase</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas putida</em> strain AJ1 – HadD</td>
<td>Barth <em>et al.</em>, (1992); Smith <em>et al.</em>, (1990)</td>
</tr>
<tr>
<td><em>Rhizobium sp.</em> – DehD</td>
<td>Leigh <em>et al.</em>, (1986); (1988)</td>
</tr>
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</table>

Class 1D dehalogenases are less common in nature than 1L enzymes. This enzyme can dehalogenate selectively D-isomeric substrates such as D-2-chloropropionic acid (D-2CP) with inversion of product configuration and only two examples are known (Table 1.1). *Pseudomonas putida* AJ1 contained two dehalogenases with HadL being specific for L-2-chloropropionic acid (L-2CP) while Had-D dehalogenated D-2CP. The native molecular mass of HadD was 135kDa (Smith *et al.*, 1990) and suggested to be tetrameric structure. The two genes, hadD and hadL from *Pseudomonas putida* AJ1 were closely linked on the genome.

In *Rhizobium sp.* the D- isomer specific enzyme is DehD. Nucleotide sequence data indicated a 29.4kDa polypeptide with 266 amino acid residues. The native molecular mass of DehD from *Rhizobium sp.* was 58 kDa suggesting a dimeric native structure. Sequence analysis comparing DehD and HadD suggested 23% amino acid identity. This was a low level of identity. Both enzymes had also no significant homology with any other dehalogenases from other classes.
Class 1L (Table 1.2) removed halide from L-2CP inverting the product configuration and react with sulfhydryl blocking reagents. This class also included halidohydrolases I and II of DCA-degrading *Pseudomonads* described by Goldman *et al.*, (1968) and the *Pseudomonas dehalogenans* NCIMB 9061 described by Little and Williams (1971). However, both these genes have not been sequenced. The native molecular mass of DehL from *Rhizobium* sp. was 60kDa whereas HadL from *Pseudomonas putida* strain AJ1 had a native molecular mass of 79kDa (Jones *et al.*, 1992). The subunit size of DehL and HadL were 31kDa and 26kDa, respectively, suggesting DehL was a protein dimer and HadL was tetramer.

*Pseudomonas species* CBS3 that grows on 4-chlorobenzoate synthesized two dehalogenases, DehCI and DehCII. Both enzymes dehalogenated L-2CP but not D-2CP. Protein analysis showed both enzymes were dimeric proteins with overall molecular masses of 41 and 64 kDa and subunit molecular masses of 28 and 29kDa respectively (Klages *et al.*, 1983; Morsberger *et al.*, 1991). When DehCI and DehCII were compared there was 45% nucleotide sequence homology. This corresponded to 38% amino-acid sequence identity and over 70% amino-acid similarity. There appeared to be a close evolutionary relationship between both *dehCI* and *dehCII* genes suggesting a common origin from an ancestral gene.

### Table 1.2. Class 1L: L-isomer specific – inverts substrate/product configuration

<table>
<thead>
<tr>
<th>Organisms – Dehalogenase</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas putida</em> strain AJ1 – HadL</td>
<td>Jones <em>et al.</em>, (1992)</td>
</tr>
<tr>
<td><em>Pseudomonas sp.</em> strain CBS3 – DehCI</td>
<td>Schneider <em>et al.</em>, (1991)</td>
</tr>
<tr>
<td><em>Pseudomonas sp.</em> strain CBS3 – DehCII</td>
<td>Schneider <em>et al.</em>, (1991)</td>
</tr>
<tr>
<td><em>Xanthobacter autotrophicus</em> strain GJ10 – DhIB</td>
<td>van der Ploeg <em>et al.</em>, (1991)</td>
</tr>
<tr>
<td><em>P. cepacia</em> strain MBA4 – Hd11IVa</td>
<td>Murdiyatmo <em>et al.</em>, (1992)</td>
</tr>
<tr>
<td><em>Moraxella sp.</em> strain B – DehH2</td>
<td>Kawasaki <em>et al.</em>, (1992)</td>
</tr>
<tr>
<td><em>Pseudomonas sp.</em> strain YL- L-DEX</td>
<td>Nardi-Dei <em>et al.</em>, (1994)</td>
</tr>
<tr>
<td><em>Rhizobium sp.</em> DehL</td>
<td>Leigh (1986); Cairns (1994)</td>
</tr>
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</table>
*Pseudomonas cepacia* MBA4 that grows on MBA (monobromoacetate) synthesised two dehalogenases III and IVa (Tsang *et al*., 1988). The DehIII was not studied in detail but the latter enzyme was found to be a class 1L enzyme (Murdiyatmo, 1991; Murdiyatmo, *et al*., 1992; Asmara *et al*., 1993). However, DehIVa was studied further with predicted 231 amino acids and molecular mass of 25.9 kDa. This value corresponded to protein observed by SDS-PAGE (23kDa). The analysis by gel filtration gave 45kDa suggesting protein dimer.

Dehalogenase DehIVa and DehCl were compared and found to have 67% amino-acid identity with 81% similarity and for DehIVa and DehClII the corresponding values were 37% and 56% amino-acid identity and similarity, respectively.

*Moraxella sp.* B produce DehH-2 was also included in Class 1L on the basis of amino acid sequence information since it showed between 40 to 50% amino-acid identity with the members of the Class 1L. However, the response to stereo-specific compounds is not known. This is an example of difficulty of classification on the basis of substrate specificity. However, the sequence data strongly suggested that this enzyme should be in class 1L.

*Pseudomonas sp.* 109 produces Deh109 which is a dimer with a slightly smaller native molecular mass (34kDa). *Pseudomonas sp.* YL also produces L-specific enzyme with native molecular mass 54kDa. Information on microbial dehalogenases in Class 1L was also found in earlier reviews of Leisinger and Bader (1993) and Slater *et al*., (1995).

Enzymes in Class 2I were distinguished by their ability to dehalogenate both D and L-isomers by a mechanism that inverts substrate product configurations (Table 1.3). The enzymes were unaffected by sulphhydryl blocking reagents which is in contrast to Class 2R. *Pseudomonas putida* PP3 was found to synthesise two 2-Haloalkanoic acid (2HAA) dehalogenases (Thomas, 1990). DehII which was previously termed fraction II dehalogenase was active against a range of halogenated compound similar to DehI. The native molecular mass of DehII was 52kDa (Topping, 1992).
### Table 1.3. Class 2I: D and L isomers as substrates – inverts substrate product configuration

<table>
<thead>
<tr>
<th>Organisms – Dehalogenase</th>
<th>References</th>
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<tbody>
<tr>
<td><em>Pseudomonas putida</em> strain PP3 – DehII</td>
<td>Weightman <em>et al.</em> (1982); Topping (1992)</td>
</tr>
<tr>
<td><em>Pseudomonas</em> strain 113 (DL-DEX)</td>
<td>Motosugi <em>et al.</em> (1982 a, b)</td>
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</table>

Motosugi *et al.*, (1982a,b) isolated *Pseudomonas species* 113 which grew on both D- and L-2CP. The dehalogenase from this organism had a native molecular mass of 68kDa and subunits of 35 kDa, suggesting it was a protein dimer. D,L-2-haloacid dehalogenase from *Pseudomonas sp.* 113, D,L-DEX, was further studied and was suggested as a new class of dehalogenase catalysing hydrolytic dehalogenation not involving enzyme-substrate ester intermediate (Nardi-Dei *et al.*, 1999). Every halidohydrolase studied so far (L-2-haloacid dehalogenase or haloalkane dehalogenase) has an active site carboxylate group that attacks the substrate carbon atom bound to the halogen atom leading to the formation of an ester intermediate. This is subsequently hydrolysed resulting in the incorporation of an oxygen atom of the solvent water molecule into the carboxylate group of the enzyme. However, the reaction mechanism of D,L-DEX shows the water directly attacks the α-carbon of 2-haloalkanoic acid to displace the halogen atom.

DehE in *Rhizobium sp.* (Allison, 1981) was found to be a non-stereospecific dehalogenase that acted on the D,L-2CP, 2,2DCP, MCA and DCA. DehE caused an inversion of configuration when chiral substrates were used. However, according to Allison *et al.*, (1983) this type of enzyme showed sensitivity to thiol reagents which was in contrast to the information by Slater *et al.*, (1997) that this class of enzyme was unaffected by this agent.
Table 1.4. Class 2R: D and L isomers as substrates – retains substrate product configuration

<table>
<thead>
<tr>
<th>Organisms – Dehalogenase</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas putida</em> strain PP3 – Dehl</td>
<td>Weightman <em>et al.</em> (1982); Topping (1992)</td>
</tr>
<tr>
<td>Isolate K37 – HdIV</td>
<td>Murdiyatmo (1991)</td>
</tr>
</tbody>
</table>

Enzymes from Class 2R differ from Class 2I enzymes in their ability to dehalogenated both D and L isomers with retention of product configuration (Table 1.4). Fraction I dehalogenase (Dehl) from *Pseudomonas putida* PP3 (Slater *et al.*, 1979, Weightman *et al.*, 1979b) was estimated to have a native molecular mass of 64 kDa. Based on the nucleotide sequence for Dehl, it was predicted to have a subunit molecular mass of 32.7 kDa, which corresponded to SDS-PAGE analysis of 33 kDa, suggesting possibly a protein dimer in the active state (Thomas 1990, Topping 1992).

Murdiyatmo, (1991) purified an enzyme from an un-identified isolate strain K37. The purified enzyme was called HdlV. The first 13 N-terminal amino acids were determined for this protein. The sequence corresponded exactly with the protein encoded by the putative Dehl open reading frame beginning at the second potential methionine residue (Slater *et al.*, 1997). Between the first and second methionine residue (the Dehl protein start) there was a strong Shine-Dalgarno sequence separated by 8 bases from the initiation codon, a distance considered to be optimal for transcription (Gold, 1988).

ii) haloalkane hydrolytic dehalogenases

Haloalkane hydrolytic dehalogenases were divided into two classes, 3R and 3B on the basis of substrate specificity. Class 3R enzymes, mainly from Gram-negative bacteria, show a restricted range of substrate specificities, Table 1.5.

*Xanthobacter autotrophicus* GJ10 was isolated and extensively studied (Janssen *et al.*, 1989, Keuning *et al.*, 1985). DhlA isolated from *Xanthobacter autotrophicus* GJ10 acted on C1 – C4 substituted alkanes such as dichloromethane, bromoethane, 1-chloropropane, 1-chlorobutane, 1,3-dichloropropane and 3-chloropropane with ethanol, 1-propanol and 1-butanol as reaction products. The enzyme was heat labile and when purified had a native molecular mass of 36 kDa and functioned without any co-factors.
The substrate affinity was in the millimolar range with a $K_m$ for dichloroethane of 1.1 mM and the pH optimum was 8.2. The reaction was strongly inhibited by thiol-blocking reagents implicating a cysteine residue at the active site (Keuning et al., 1985).

The nucleotide sequence determined by Janssen et al., (1989) predicted a 310 amino acid protein with molecular mass of 35.1 kDa. This suggested the native enzyme was composed of one polypeptide subunit. The dehalogenase was crystallised and the three dimensional protein structure determined (Franken et al., 1991). The enzyme was composed of two domains. The main one with an $\alpha/\beta$ structure which is common to many hydrolytic proteins of the general $\alpha/\beta$ hydrolase type. The second domain was composed of five $\alpha$ helices linked by loops. The active site was a hydrophobic cavity located between the two domains. The three key amino acids involved in catalysis are Asp124, His289 and Asp260. The importance of Asp124 in the active site was revealed by site directed mutagenesis where replacement of Asp124 with alanine, glycine or glutamic acid inactivated the enzyme (Pries et al., 1994).

The N-terminal amino acid analyses of haloalkane dehalogenases from Anicylobacter aquaticus strains AD20 and Xanthobacter autotrophicus GJ11 showed that they were identical to the dichloroethane dehalogenase from X. autotrophicus GJ10 (Janssen et al., 1985).

LinA and LinB from Sphingomonas paucimobilis UT26 are two different types of dehalogenase localised in the periplasmic space. These two enzymes are the first report on the subcellular localization of dehalogenases that are involved in the degradation of halogenated xenobiotics in Gram-negative bacteria (Nagata et al., 1999).

A newly isolated Mycobacterium tuberculosis H37Rv also has the ability to convert haloalkanes to their corresponding alcohols by a hydrolytic mechanism (Jesenska et al., 2000). At least 4 halogenated alkanes such as 1-chlorobutane, 1-chlorodecane, 1-bromobutane, and 1,2-dibromoethane could react with the dehalogenase from this organism. The amino acid sequence of haloalkane dehalogenase is known for Xanthomonas autotrophicus GJ10 (DhlA), Sphingomonas paucimobilis UT26(LinB) and Rhodococcus NCIMB 13064 (Dha) (Kulakova, et al., 1997). These three sequences were compared to the complete genome of Mycobacterium tuberculosis H37Rv (Cole...
et al., 1998) and revealed the presence of 3 different genes encoding putative haloalkane dehalogenases in the genome of this organism.

**Table 1.5. Class 3R: Restricted range of substrates**

<table>
<thead>
<tr>
<th>Organisms – Dehalogenase</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>DhlA</td>
<td></td>
</tr>
<tr>
<td><em>Ancylobacter aquaticus</em> strains AD20 and AD25</td>
<td>van den Wijngaard et al., (1992)</td>
</tr>
<tr>
<td><em>Xanthobacter autotrophicus</em> strain GJ11</td>
<td>van den Wijngaard et al., (1992)</td>
</tr>
<tr>
<td><em>Sphingomonas paucimobilis</em> strain UT26 – Lin A; Lin B</td>
<td>Nagata et al., (1993a,b)</td>
</tr>
</tbody>
</table>

In Class 3B (Table 1.6) were haloalkane dehalogenases isolated from various *Rhodococci* and closely related Gram-positive bacteria like *Rhodococcus erythropolis* strain Y2 (Sallis et al., 1990). *Anthrobacter sp.* HA1 utilised 18 different 1-chloro, 1-bromo and 1-iodoalkanes for growth but not 1-flouroalkanes (Scholtz et al., 1987). There are three dehalogenases produced by this organism. One of the dehalogenases (1-chlorohexane dehalogenase) is a monomeric protein with a molecular mass of 37 kDa and pH optimum of 9.5.

The hydrolytic dehalogenase from *Corynebacterium species* m15-3 was a single polypeptide with a molecular mass of 33-36kDa. This enzyme was similar to the hydrolytic dehalogenase from *Arthrobacter species* HA1 and *Rhodococcus erythropolis* Y2 with an ability to hydrolyse C2 to C12 mono and disubstituted alkanes (Sallis et al., 1990). Further support for this Class 3B was that the first 20 N-terminal amino acids from these enzymes were identical but had no sequence homology with the haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10.
1.5 Regulation of gene expression

1.5.1 RNA Polymerase Sigma (σ) factor

The production of mRNA and protein by a cell is an energy-intensive process, which it is therefore necessary to control by preventing unnecessary gene expression. A number of control mechanisms have been identified such as repression and activation of transcription through the binding of specific proteins to the promoter region.

The ability of RNA polymerase to recognise the promoter region is varied. The RNA polymerase of *E. coli* is the best characterised and it consists of a complex enzyme with five core subunits (α₂ββ′ω; mw: 390,000Da) and a sixth subunit designated as σ subunit. The σ subunit is commonly designated by its molecular weight and binds to the core and directs the enzyme to specific binding sites on the DNA. These six subunits together are also referred to as the RNA polymerase holoenzyme (Nelson and Cox, 2000).

Most *E. coli* promoters are recognised by a single sigma subunit called σ\(^{70}\) (the superscript refers to the apparent molecular weight (kDa) on SDS-PAGE). The σ\(^{70}\) enables the holoenzyme to initiate transcription from the −35/-10 type promoter (Helmann and Chamberlain, 1988). These sequences are important interaction sites for the σ\(^{70}\) subunit. Although the sequences are not identical for all bacterial promoters in the same class, certain nucleotides that are particularly common at each position lead to a consensus sequence.

*E. coli* has other classes of promoters with different σ factors. The best characterised of these minor factors is σ\(^{32}\), the heat shock transcription factor. *E. coli* produces 17
proteins in response to a variety of stressful conditions including high temperature and phage infection (Neidhardt et al., 1984; Watson, 1990). At high temperature heat shock proteins are made at higher levels. RNA polymerase binds to the promoters of these genes by $\sigma^{32}$ rather than by $\sigma^{70}$. By using different $\sigma$ factors the cell can coordinate the expression of sets of genes that allow the desired changes of the cell physiology. Repressor proteins bind to a specific site of the operator sequence that is generally close to the promoter. RNA polymerase holoenzyme that is capable of initiating the transcription from the appropriate promoter is blocked by this activity (Collado-Vides et al., 1991). Regulation by means of a repressor protein that blocks transcription is referred to as negative regulation and an activator that binds to a specific site of the DNA that enhances the activity of RNA polymerase leads to positive regulation.

$\sigma^{54}$ does not show homology to $\sigma^{70}$ or to any of the minor sigma factors initiating from -35/-10 type promoter (Thony and Hennecke, 1989). $\sigma^{54}$ is required for diverse functions such as nitrogen fixation (Gussin et al., 1986) and catabolism of toluene and xylene (Inouye et al., 1987; Kohler et al., 1989). The expression of a gene dependent on $\sigma^{54}$ has been demonstrated in a variety of organisms: E.coli (Hirschman et al., 1985; Pseudomonas putida (Kohler et al., 1989); Rhizobium meliloti (Ronson et al., 1987) and Agrobacterium tumefaciens (Wu et al., 1992). The $\sigma^{54}$ recognises the minimal consensus sequence promoter -24(GG)/-12(GC) (Johnston and Downie, 1984; Ausubel, 1984; Kustu et al., 1989). However, the binding of this holoenzyme to the promoter region is not able to melt the DNA duplex and initiate RNA synthesis without the presence of an activator protein bound upstream of the promoter (Popham et al., 1989; Reitzer and Magasanik, 1986). This is in contrast to the $\sigma^{70}$ which is able to initiate transcription alone and is subject to control by interaction with operators bound close to the promoter (Collado-Vides et al., 1991). All the $\sigma^{54}$ dependent promoters have been found to be positively controlled (Collado-Vides et al., 1991). The polymerase must be controlled in order to provide regulation. Production of $\sigma^{54}$ is constitutive (Ronson et al., 1987; Kustu et al., 1989) and the activator protein is the principal regulatory component. However, the activator protein requires modification to ensure that the activation only occurs when it is required. For NtrC, the activator of a variety of nitrogen assimilation functions, the protein produced is not in an active form.
It can be activated by phosphorylation by a protein kinase, NtrB, only under conditions of nitrogen limitation (Keener and Kustu, 1988).

Role of $\sigma^{54}$ dependent activation is quite distinct from that of $\sigma^{70}$. It has been noted that this type of regulation has much in common with eukaryotic systems and it has been suggested that the $\sigma^{54}$ system represents an evolutionary link between the two systems (Collado-Vides et al., 1991).

### 1.5.2 Regulation of transcription

Transcription factors can be recognised as families of related proteins for example LysR family (Schell, 1993), the AraC family (Gallegos et al., 1993), the $\sigma^{70}$ family (Lonetto et al., 1992), and the Y Box family (Wolffe, 1994). These families are found in *E.coli* and *S. typhimurium*.

The LysR family consists of more than 50 different transcriptional activators and repressors that share a highly conserved N-terminal DNA-binding domain consisting of a helix-turn-helix motif and flanking sequences. However, in this family the less-conserved region is the C-terminal region that has a sensory function (Kullik et al., 1995). The transcription regulation from this family is likely to be due to a change in the DNA-binding properties associated with the binding of the co-inducer. In several cases it has been shown that in the absence of the co-inducer the LysR type of regulator binds to DNA sequences at position −65 relative to the transcriptional start site of the regulated promoter. In the presence of co-inducer, additional contacts with DNA are observed near position −35 of the DNA is observed (Schell, 1993).

The AraC family consists of 27 proteins sharing a characteristic helix-turn-helix–DNA-binding motif near the C-terminus (Gallegos et al., 1993; Ramos et al., 1990). The N-terminal region is poorly conserved and mutation at this portion alters the specificity for the co-inducer.

The $\sigma^{70}$ family protein contains 4 major conserved regions. Regions 1 and 2 may constitute an N-terminal domain and regions 3 and 4 constitute a C-terminal region. Several functions have been assigned to region 2, including the binding of the core
polymerase, contact with DNA sequences near position –10 relative to the site of transcription initiation, and melting of the DNA strands surrounding the site of initiation. Region 4 contains a helix-turn-helix DNA-binding motif and is involved in recognition of DNA sequences at position –35.

The activation of transcription from a promoter recognised by $\sigma^{54}$ is different from $\sigma^{70}$ form of RNA polymerase. Transcription by $\sigma^{54}$ RNA polymerase requires an accessory transcriptional activator. The protein that binds to the $\sigma^{54}$ dependent promoters has an ATPase activity that is required for transcriptional activation. $dehR_I$ from Pseudomonas putida PP3, was reported to have a significant similarity to other $\sigma^{54}$ dependent activators, for example XylR activator from Pseudomonas putida (Inouye et al., 1987) (which regulates the TOL plasmid xyl-regulon) where the central region showed good degree of similarity. This region is well conserved in many $\sigma^{54}$ dependent activator proteins (Drummond et al., 1986) for example DmpR which regulates the dmp operon associated with dimethly phenol catabolism in Pseudomonas putida (Shingler et al., 1993). It is proposed that the N-terminal of this protein is responsible for environmental sensing to activate initiation of transcription. The central domain is responsible for interactions with the polymerase and ATP binding (Thöny and Hennecke, 1989).

The Y-Box family share a highly conserved N-terminal domain involved in the recognition of nucleic acids; depending on the protein, RNA, double stranded DNA, or single-stranded DNA may be bound. There are two examples in prokaryotes, in E.coli transcriptional activator of the cold shock regulon, designated CS7.4 (Goldstein et al., 1990) and a similar protein from Bacillus subtilis designated CspB (Willimsky, et al., 1992). Both of these prokaryote homologs consist of highly conserved nucleic acid-binding domain.

Expression of many genes is regulated. There are two main types of gene expression in E.coli. Negative control is defined as the inhibition of gene expression at the level of transcription initiation by a regulatory protein, described as a repressor protein (Neidhardt, 1996). The specific repressor protein produced by the regulator gene is active in the absence of the inducer in completely blocking the synthesis of mRNA.
When inducer is added, it combines with the repressor protein and inactivates the repressor protein. Inhibition of mRNA synthesis is overcome and the enzyme can then be made. This type of control was observed in the lac operon where the lac promoter is part of the operon which is comprised of three structural genes Z, Y and A, coding for β-galactosidase, lactose permease and thiogalactoside transacetylase, respectively. The inducer molecule, allolactose or another suitable inducer like IPTG, is required because in its absence, the repressor protein (the lacI gene product), binds to the lac operator (lacO), thus preventing the binding of RNA polymerase to the lac promoter (lacP). When inducer is present it will bind to the repressor and alter its conformation which releases it from lacO thereby allowing RNA polymerase to initiate transcription (Figure 1.2).

Another type of control is called positive control. In positive control a regulator protein promotes the binding of RNA polymerase, thus acting to increase mRNA synthesis. E.coli can take up and catabolise the pentose L-arabinose. The arabinose –specific proteins encoded by these genes are AraC, the arabinose-responsive transcription activator protein. AraBAD, three arabinose inducible enzymes that convert L-arabinose to D-xylulose-5-phosphate, an intermediate in the pentose phosphate shunt (Englesberg et al., 1965).

The arabinose operon includes the araC gene encoded regulatory C protein, two operator sites araO₁ and araO₂, a binding site for CAP protein and another regulatory site, araI (Figure 1.3). The arabinose operon is under a dual control. The C protein also serves as a negative control of araC. Transcription of araC is in the opposite direction to that of the structural genes araB,A,D and is controlled by araO₁ rather than the araO₂ operator site. mRNA for the C protein (regulator protein) is formed when the level of C protein and cAMP-CAP is low. When C protein is abundant transcription of the C gene is stopped because C protein binds to araO₁. Thus, transcription of C protein is autoregulated. The binding of a second molecule of C protein to araO₂ blocks the synthesis of araB,A,D mRNA by forming a DNA loop that also bind to araI, which is adjacent to the promoter for the araB,A,D genes. Looping also blocks the binding of RNA polymerase. The DNA loop is not formed when cAMP-CAP is abundant and arabinose is bound to the C protein.
Figure 1.2. The process of enzyme induction involving a repressor

a) A repressor protein binds to the operator region and blocks access for the RNA polymerase

b) An inducer binds to the repressor and inactivates the repressor protein

Transcription by RNA polymerase occurs and mRNA is formed. In \( lacZYA \) operon or \( lac \) operon, the repressor is the \( lac \) repressor (\( lacI \)), and the inducer is allolactose

Figure 1.3. Arabinose operon positive control system and its regulator gene
However, the presence of positive regulatory factors enables RNA polymerase to bind to the promoter site for the \textit{araB,A,D} genes for transcription of the gene. In this case mRNA for C protein is not formed because \textit{araO} is occupied by C protein.

Another example for positive control is the maltose operon. \textit{E.coli} can grow on maltose by taking the sugar and cleaving it to release glucose and glucose 1-phosphate. MalT is a positive regulator of the maltose genes. The maltose activator protein (MalT) cannot bind to the DNA unless it first binds maltose. When the protein binds to the DNA it allows RNA polymerase to begin transcription. Activators like repressors recognise specific sequences on the DNA. The sequence that serves as the binding site for the activator is called an activator binding site (Figure 1.4). The genes needed for maltose utilisation are located in several operons with an activator binding site on each operon.

1.5.3 Dehalogenase gene organization

Reports on the genetic organisation of dehalogenases have been infrequent. Generally, most of the dehalogenase systems isolated are inducible (Allison \textit{et al}., 1983; Bollag and Alexander, 1971; Hardman and Slater, 1981a; Brokamp and Schmidt, 1991) although constitutively produced dehalogenases have also been described (Janssen \textit{et al}., 1994; Kawasaki \textit{et al}., 1981b).

The genes of catabolic pathways are sometimes plasmid-encoded as described for two haloacetate dehalogenases by Kawasaki \textit{et al}., (1981b). Plasmid pUOI encoding haloacetate dehalogenase H-1, H-2 and mercuric reductase was isolated from \textit{Moraxella sp.} strain B with the size of the plasmid estimated to be 65.55kb (Kawasaki \textit{et al}., 1981c). The loss of H-2 function in pUOII was correlated with the loss of 4.5kb from pUO1. Both pUO1 and pUOII are transmissible to \textit{Pseudomonas} and \textit{E.coli} and express their function in both hosts (Kawasaki \textit{et al}., 1981d; 1983a,b). The H-1 gene was located within a 1.6kb region and the H-2 gene within a 2kb region of plasmid pUO1 (Kawasaki \textit{et al}., 1984). The pUOI plasmid was not associated with any transposable element (Kawasaki \textit{et al}., 1985).
Figure 1.4. The maltose positive control system

a) In the absence of inducer, activator protein and RNA polymerase cannot bind to the DNA.

b) An inducer molecule binds to the activator protein, which then binds to the activator binding site. This allows RNA polymerase to bind to the promoter and begin transcription. In the malEFG operon, the activator protein is the maltose activator protein and the inducer is the sugar maltose.
Weightman et al., (1979a;b) showed that *Pseudomonas putida* PP3 produced two dehalogenases (Dehl and Dehll) that could be separated on a DEAE-Sephadex A50 column and distinguished by electrophoretic mobility on non-denaturing PAGE. However, Dehl was studied in greater detail (Thomas, 1990). The gene encoding gene Dehl (*dehl*) was located on a mobile DNA element, which could be inserted into target plasmids at high frequency and subsequently transferred from these plasmids into the chromosome of another *P.putida* strain. The mobile element was designated as DEH. A DEH copy inserted into the EcoRI-G restriction fragment of TOL plasmid pWWO was cloned and the position of *dehl* was mapped. Expression of this gene was shown to be under positive control of an adjacent regulatory gene (*dehR*). Partial sequence analysis of these two genes indicated that the regulatory protein belongs to a group of RNA polymerase $\sigma^{54}$ dependent activator proteins (Topping et al., 1995).

Janssen et al., (1985) reported that *Xanthobacter autotrophicus* GJ10 produces two different dehalogenases, an haloalkane dehalogenase and an haloalkanoic acid dehalogenase, which is active with halogenated aliphatic carboxylic acids (Keuning et al., 1985; van der Ploeg et al., 1991). The gene encoding the haloalkanoic acid dehalogenase (*dhlB*) is chromosomally located (Tardiff et al., 1991) and has been cloned and sequenced (van der Ploeg et al., 1991). The DNA sequence upstream of *dhlB* carried two open reading frames, which can be expressed in *E.coli*. In addition, sequence comparison with other sequences in the database suggested that these genes may have a function in transport of haloacids (*dhlC*) and regulation of expression of *dhlB* (*dhlR*) (van der Ploeg and Janssen, 1995). Upstream of *dhlC*, a $-24/-12$ promoter sequence was found associated with $\sigma^{54}$ dependent activator protein. The genetic organisation of the *dhlB* region suggested that expression of *dhlC* and *dhlB* was controlled by the product of *dhlR* and $\sigma^{54}$. However, no further attempts were carried out to characterise the regulator gene product.

*Alcaligenes xylosoxidans* ABIV isolated from soil was able to utilise 2-haloalkanoic acids including D,L2-CP and 2,2DCP (Brokamp and Schmidt, 1991). Dehalogenase from this organism was plasmid encoded (pFL40) as demonstrated by the transfer of the *dhlIV* halidohydrolase gene from *Alcaligenes xylosoxidans ssp. denitrificans* strain ABIV to indigenous soil bacteria in a soil microcosm (Brokamp and Schmidt, 1991).
The cloning of 6.5kb from pFL40 into pUC18 resulted in plasmid pFL45, which included a putative regulator gene \textit{dhlRIV} and \textit{dhllV} that enabled \textit{E.coli} RRI transformed with this plasmid to grow on MCA, D,L2-CP, and 2,2DCP minimal medium. Like the parent strain this construct shows inducible expression of the halidohydrolase. The \textit{dhllV} gene is likely to exhibit positive regulation only in the presence of halogenated substrate where dehalogenase activity was detected in crude extract. When succinic acid or glucose was used in the growth medium no dehalogenase activity was detected. Further study of pFL40 by subcloning into pUC18 or pUC19 suggested that \textit{dhlRIV} is necessary for controlling \textit{dhllV} production. A construct without the regulator gene did not express \textit{dhllV} but dehalogenase enzyme was produced constitutively in the presence of the \textit{lac} promoter system (Brokamp \textit{et al.}, 1997). The amino acid sequence of \textit{dhlR} from pFL45 was compared to \textit{dehR}\textsubscript{i} from \textit{Pseudomonas putida} PP3 (Topping \textit{et al.}, 1995) and resulted in sequence similarities of 95%. A putative -12/-24 promoter sequence was detected in the upstream region of \textit{dhllV} with a proposed function as a $\sigma$\textsuperscript{54}-dependent activator protein binding site. However, no further attempt to characterise the \textit{dhlRIV} gene product was described.

1.6 \textbf{Identification of organisms by 16S rRNA sequencing}

The comparison of 16S rRNA sequences is of particular importance in estimating the phylogenetic relatedness of bacteria. In the past identification of organisms was largely based on biochemical and morphological studies. 16S rRNA sequencing comparison is currently favoured because advances in molecular techniques have yielded a library of oligonucleotide rRNA probes that are highly specific for particular bacterial taxonomic groupings (Hopkins and Macfarlane, 2000).

Ribosomes are a useful target because they are universal with a conserved function and so allows comparisons between different organisms. The 16S rRNA is particularly well studied because unlike 5S rRNA it is large enough to give a useful amount of information and it is preferred to the 23S rRNA due to its smaller size making it easier to obtain a complete sequence (Fox \textit{et al.}, 1977). According to Lane \textit{et al.}, (1985) the simplest method currently in use is the amplification of the gene encoding the 16S rRNA followed by sequencing of the PCR product.
The more highly conserved regions are the basis for the phylogenetic analysis (Woese, 1987) and for the design of universal oligonucleotide primers for the amplification and identification. However, a problem with commonly used primers is that they have been constructed theoretically using an incomplete database of 16s rRNA sequences (Marchesi et al., 1998).

1.7 Oligotrophy
A less well understood part of the microbial community in soil and in other natural environments are the oligotrophs. Generally these microorganisms can be maintained on media containing less than 15mg/l of organic matter. The study of oligotrophs and slow-growing organisms is an important area in environmental microbiology (Prescot et al., 1996). Oligotrophs are ubiquitous in the environment and have been isolated from soil, rivers, lakes, oceans and tap-water lacking organic substances. There are two types of oligotrophs that have been identified. Those that are able to grow only on a very low concentration of carbon are called obligate oligotrophs and those that are able grow at both low and high concentrations of organic substances are called facultative oligotrophs.

A detailed study was carried out on the enteric bacterium Enterococcus faecalis. The natural habitat of this bacterium is in the intestine of humans and animals. When discharged into a natural aquatic system this microorganism needs to adapt for survival in a hostile environment. The ability of Enterococcus faecalis to survive in an oligotrophic environment such as in tap water was shown (Hartke et al., 1998).

Many oligotrophic bacteria can be isolated from clinical materials including urine, sputum and vaginal discharges and throat swabs. However, the clinical significance of such oligotrophic bacteria is uncertain (Tada et al., 1995).

Slow growth of oligotrophic bacteria was possibly due to the slow process of transport of nutrients into the cell. Dissolved nutrients entering the aquatic environment are assimilated into microorganisms due to permeability and active transport through the cytoplasmic membrane. In some oligotrophic bacteria there is a cellular appendage (prostheca) whose function is to increase the surface area of cell and cytoplasmic membrane for increasing transport of substances into a cell. A biochemical study on
prostheca of *Asticcacaulis biprosthecum* revealed that it did not contain DNA, RNA, NADH-oxidase and NADH-dehydrogenase. This highlights the specialised function of prostheca connected with the active transport of nutrients (Jordan et al., 1974). Some oligotrophic bacteria show inhibition of growth in rich nutrient media (Kuznetsoz et al., 1979). One reason may be the action of toxic products of metabolism particularly hydrogen peroxide which forms in a number of metabolic reactions. Dubinina (1977) showed that the oligotrophic bacterium *Leptothrix pseudoochraceae* begins to lyse due to accumulation of hydrogen peroxide in a rich medium. The addition of catalase resulted in growth of this organism.

Xenobiotic chemicals that enter the environment are mostly from chlorinated aliphatic acid compounds. Many of these pollutants are present in natural waters only at very low concentrations. However, such concentrations are still sufficient to be a public health and environmental concern (Fauzi et al., 1996). Biodegradation of low substrate concentrations of xenobiotic compounds has been a neglected field of research. The rate of biodegradation at low concentration usually decreases with lower initial substrate concentrations (Boethling and Alexander, 1979). For example the herbicide (2,4-dichlorophenoxy acetic acid) at 2.2 parts/billion was mineralised more slowly in stream water than at higher concentrations (22 ppm). In addition, the carbon of very low concentrations of xenobiotics is often not converted to biomas carbon, thus other carbon sources may be required for cell growth under these conditions (Subba-Rao et al., 1982).

The bacterial degradation of low concentrations of 1,3-dichloro-2-propanol (1,3-DCP) has been reported. The isolate was identified as *Agrobacterium sp.*, which was able to dehalogenate 1,3-DCP with a Km value of 0.1mM (Fauzi et al., 1996). However, there is no report so far in the current literature for degradation of low concentration of haloalkanoic aliphatic acids.

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1.8 Biochemistry and genetics of *Rhizobium sp.* dehalogenases

Berry *et al.*, (1979) isolated from soil a *Rhizobium sp.* able to grow in mineral salt medium containing 2,2DCP as sole carbon source. The organism can also grow on D,L2-CP but not on 3CP. Non-halogenated compounds such as pyruvate and compounds degraded via it such as lactate, glucose and glycerol were also good substrates for growth. The product formed when crude extracts of 2,2DCP-grown bacteria reacted with that compound was pyruvate with concomitant release of 2 chloride ions for each molecule of pyruvate formed. Compounds such as MCA, DCA and TCA were not utilised for growth but they are good substrates for the dehalogenase enzymes. These substrates were not utilised for growth presumably because the dehalogenation product was not used as a sole carbon source by this bacterium. Inducers for dehalogenase enzymes were also identified when growing *Rhizobium* on a non-halogenated carbon source. A wide range of haloalkanoates, which induced dehalogenase activity in cells growing on lactate are summarised in Table 1.7.

Dehalogenases of *Rhizobium sp.* were termed DehL (dehalogenase I), DehE (dehalogenase II) and DehD (dehalogenase III). DehL was stereospecific for L-2CP and DehD was a D-2CP specific dehalogenase. DehE was non-stereospecific and acted on D,L-2CP and 2,2DCP. For each dehalogenase the lactate produced from D,L-2-CP has the opposite stereochemical form to that of the substrate. All three dehalogenases are compared as in Table 1.8.
Table 1.7. The effects of halogenated compounds on *Rhizobium sp.* dehalogenase enzymes

<table>
<thead>
<tr>
<th>Compound</th>
<th>Substrate for growth</th>
<th>Substrate for enzyme</th>
<th>Inducer for enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,2-dichloropropionic acid (2,2DCP)</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>D,L2-chloropropionic acid (D,L2-CP)</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>D,L2-bromopropionic acid (D,L2-BP)</td>
<td>NO</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>2,3-dichloropropionic acid (2,3DCP)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dichloroacetate (DCA)</td>
<td>NO</td>
<td>YES</td>
<td></td>
</tr>
<tr>
<td>Trichloroacetate (TCA)</td>
<td>NO</td>
<td>YES</td>
<td></td>
</tr>
<tr>
<td>Dibromoacetate (DBA)</td>
<td>NO</td>
<td>YES</td>
<td></td>
</tr>
<tr>
<td>Tribromoacetate (TBA)</td>
<td>NO</td>
<td>YES</td>
<td></td>
</tr>
<tr>
<td>3-chloropropionic acid (3CP)</td>
<td>NO</td>
<td>NO</td>
<td>YES</td>
</tr>
<tr>
<td>3-bromopropionic acid (3BP)</td>
<td>NO</td>
<td>NO</td>
<td>YES</td>
</tr>
</tbody>
</table>

Table 1.8. Comparison of physical and biochemical properties of *Rhizobium sp.* haloalkanoic acid dehalogenases (Leigh *et al.*, (1986,1988); Cairns *et al.*, (1996) and Stringfellow *et al.*, (1997).

<table>
<thead>
<tr>
<th>Dehalogenase/ regulation</th>
<th>Substratespecificities</th>
<th>Sub-unit (kDa)</th>
<th>pH Optimum</th>
</tr>
</thead>
<tbody>
<tr>
<td>DehL/ Inducible</td>
<td>L-2CP; DCA</td>
<td>31</td>
<td>8.4</td>
</tr>
<tr>
<td>DehE/ Inducible</td>
<td>D,L-2CP; 2,2-DCP; DCA, TCA, MCA</td>
<td>32</td>
<td>6.1-10.5</td>
</tr>
<tr>
<td>DehD/ Inducible</td>
<td>D-2-CP; MCA</td>
<td>29</td>
<td>9.1-10.5</td>
</tr>
</tbody>
</table>
1.8.1 Regulation of *Rhizobium sp.* dehalogenase synthesis

*Rhizobium sp.* was reported to make three dehalogenases. It was curious why this organism produced more than one dehalogenase when DehE could act on all the substrates that DehL and DehD could act on. *Rhizobium sp.* dehalogenases were studied at a genetic level using a series of mutant strains. The mode of regulation of the dehalogenases was proposed from this study by Leigh *et al.*, (1986).

A mutant **Type A** produced by chemical mutagenesis lacked the ability to utilise either 2,2DCP or D,L2-CP as sole carbon and energy source. Enzyme assays and polyacrylamide gel electrophoresis did not show the presence of any dehalogenases.

Secondary mutants were then selected from this strain by plating onto either 2,2DCP or D,L2-CP. This gave rise to three types of secondary mutant strains. When using 2,2DCP as the selective media, two types of secondary mutants arose, **Types 1** and **2**. **Type 1** regained inducible production of the dehalogenases i.e. wild type phenotype, with all three dehalogenases being made inducibly. **Type 2** constitutively produced DehE without forming DehL and DehD. Using D,L2-CP as the selective media a mutant strain that constitutively produced DehL and DehD (**Type 3**) but without DehE was found. The characteristics of these mutants are summarised in Table 1.9. From the data obtained, it was possible to suggest a model for the regulation of the dehalogenase gene expression (Figure 1.5).

**Table 1.9. ***Rhizobium sp.* dehalogenase synthesis

<table>
<thead>
<tr>
<th>Mutant Strains</th>
<th>DehL</th>
<th>DehE</th>
<th>DehD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Type A</strong></td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td><strong>Type 1</strong></td>
<td>Made inducibly</td>
<td>Made inducibly</td>
<td>Made inducibly</td>
</tr>
<tr>
<td><strong>Type 2</strong></td>
<td>Absent</td>
<td>Made constitutively</td>
<td>Absent</td>
</tr>
<tr>
<td><strong>Type 3</strong></td>
<td>Made constitutively</td>
<td>Absent</td>
<td>Made constitutively</td>
</tr>
</tbody>
</table>
The **Type A** mutant was proposed to carry a mutation in the regulator gene. This will cause the loss of production of all the dehalogenases provided that all three genes are controlled by this regulator. To obtain **Type 1** secondary mutant (wild type phenotype) required either reversion of the original mutation in the regulator gene, or a repressor mutation in the regulator gene. **Type 2** secondary mutant produced DehE constitutively. For this to occur, a mutation in the promoter region (P1) results in production of DehE enzyme independent of the regulator protein. So only the gene encoding DehE enzyme was transcribed. The P2 region is not mutated, so that DehD and DehL would still not be produced.

The **Type 3** secondary mutant gave constitutive expression of DehD and DehL. This is due to a similar reason to that for the **Type 2** mutant, but there is now a mutation in the promoter region P2, controlling expression of both DehD and DehL.

To date the proposed relative location of \textit{dehD} and \textit{dehL} genes has been confirmed by sequence analysis. \textit{dehD} was located upstream of \textit{dehL} with 177bp of non-coding DNA between them (Cairns et al., 1996). The third Rhizobial dehalogenase, \textit{dehE}, was also sequenced. However, this gene is not particularly close to \textit{dehL} and \textit{dehD} and its relative location to them was not known. Upstream of \textit{dehE} was found a second truncated ORF containing part of a putative dehalogenase regulatory gene (\textit{dehR}). The deduced amino acid sequence showed a significant identity of 51% and similarity of 74% (when conservative substitutions were taken into account) to the N-terminal region of a \textit{Pseudomonas putida} dehalogenase regulatory gene product suggesting that a regulatory gene (\textit{dehR}) was located close to \textit{dehE} (Stringfellow et al., 1997).

The location of these dehalogenase genes supported the genetic organisation proposed by Leigh (1986) (Figure 1.5). However, this proposal must be further investigated by isolating the regulatory gene and establishing whether a single regulator gene controls all three dehalogenases.
Figure 1.5. Proposed genetic organisation and regulation for the *Rhizobium sp.* dehalogenase genes

R: Regulator gene; controls all three dehalogenases  

*P1/P2*: Promoter regions  

*dehE, dehD, dehL*: Structural genes for dehalogenases  

Arrows: indicate sites of mutations

**Type A mutant:** No dehalogenases detected  
**Type 1 mutant:** Reversion to wild type  
**Type 2 mutant:** Constitutive production of DehE only  
  (mutation in promoter P1)  
**Type 3 mutant:** Constitutive production of DehL and DehD only (mutation in promoter P2)
1.9 Aims

*Rhizobium sp.* was known to produce at least three dehalogenases with one of the dehalogenases (DehE) able to act on all the identified substrates of the other two (DehL and DehD). The aim of the present study was to investigate possible reasons for the production of multiple forms of dehalogenase. For this purpose, various kinetic properties (Km, Kcat and Specificity Constant) for the three known dehalogenases using between them fourteen different halogenated substrates were determined. The ability of the *Rhizobium sp.* to grow at low halogenated acid concentration was investigated to see whether any additional dehalogenases(s) were produced. In addition, a putative regulator gene that had been suggested to control expression of the three known dehalogenase structural genes was cloned and the function of its product investigated.
CHAPTER 2

MATERIALS AND METHODS
2.1 Bacterial strains and vectors

Bacterial strains and plasmids used in this study are given in Tables 2.1 and 2.2, respectively. The vector pUC18 was used for general cloning (Figure 2.1). This vector is a high copy number plasmid, which confers resistance to ampicillin at a concentration of 100 \( \mu g/ml \). The plasmid also contains \textit{lacZ'} that codes for a fragment of \( \beta \)-galactosidase that allows blue/white screening for inserted DNA with a host containing a chromosomal fragment \textit{lacZAM15} which codes for the remaining fragment of \( \beta \)-galactosidase.

\[\text{Figure 2.1. Restriction map of the general purpose cloning vector pUC18/19}\]
Table 2.1. Bacterial strains used in the course of this study

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>GENOTYPE</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> K-12</td>
<td><em>recA1 supE44 endA1 hsdR17 gyrA96 relA thiΔ(lacproAB)F' [traD36p roAB</em> lacF' lacZΔM15]*</td>
<td>Yanisch-Peron <em>et al.</em>, (1985)</td>
</tr>
<tr>
<td>JM 109</td>
<td><em>hsdS gal (NclIts857 ind 1 Sam7 nin 5 lacUV5-T7 geneI)</em></td>
<td>Studier <em>et al.</em>, (1986)</td>
</tr>
<tr>
<td><em>Escherichia coli</em> BL21 (DE3)</td>
<td><em>supIΔ(lac-proAB)hsd5F' [proAB</em> lacF' lacZΔM15]*</td>
<td>Gough and Murray (1983)</td>
</tr>
<tr>
<td><em>Escherichia coli</em> K-12</td>
<td><em>tetRA(mcrA)183Δ(mcrCB-hsdSMR-mrr) 173endA1 supE44 thi-1 recA1 gyrA96 relA lac Hte [F'proAB lacF'ZΔM15 Tn10(TetR)Amy CamR]</em></td>
<td>Bullock, <em>et al.</em>, (1987)</td>
</tr>
<tr>
<td>NM522</td>
<td></td>
<td>Greener and Jerpseth (1993)</td>
</tr>
<tr>
<td><em>Escherichia coli</em> K-12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XL10 Gold supercompetent (Stratagene)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2.2. Plasmids used in the course of this study

<table>
<thead>
<tr>
<th>PLASMIDS</th>
<th>ORIGINAL VECTOR</th>
<th>RELEVANT FEATURES</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUC 18/19</td>
<td></td>
<td>amp R, lac Z'</td>
<td>Yanisch-Peron et al., (1985)</td>
</tr>
<tr>
<td>pT7-7</td>
<td></td>
<td>T7RNA promoter</td>
<td>Tabor et al., (1985)</td>
</tr>
<tr>
<td>pET Xa/LIC</td>
<td></td>
<td>T7RNA promoter</td>
<td>Aslandis and de Jong, (1990); Haun et al., (1992)</td>
</tr>
<tr>
<td>pJS 771</td>
<td>pT7-7</td>
<td>dehE'</td>
<td>Stringfellow et al., (1997)</td>
</tr>
<tr>
<td>pSC3</td>
<td>pUC18</td>
<td>dehD'</td>
<td>Cairns (1994)</td>
</tr>
<tr>
<td>pSC4</td>
<td>pUC18</td>
<td>dehL'</td>
<td>Cairns (1994)</td>
</tr>
<tr>
<td>pFH45</td>
<td>pUC18</td>
<td>dehR' (incomplete)</td>
<td>This study</td>
</tr>
<tr>
<td>pFH648</td>
<td>pUC18</td>
<td>dehE', dehR' (incomplete)</td>
<td>This study</td>
</tr>
<tr>
<td>pFH772</td>
<td>pT7-7</td>
<td>dehR'</td>
<td>This study</td>
</tr>
<tr>
<td>pFH18</td>
<td>pUC18</td>
<td>dehR'</td>
<td>This study</td>
</tr>
<tr>
<td>pHCl</td>
<td>pUC18</td>
<td>dehE', dehR' (incomplete)</td>
<td>This study</td>
</tr>
<tr>
<td>pFH100</td>
<td>pUC18</td>
<td>dehR' (incomplete)</td>
<td>This study</td>
</tr>
<tr>
<td>pHC2</td>
<td>pUC18</td>
<td>dehE', dehR'</td>
<td>This study</td>
</tr>
<tr>
<td>pHC773</td>
<td>pT7-7</td>
<td>dehD', dehL', dehR'</td>
<td>This study</td>
</tr>
<tr>
<td>pHC773-ΔP</td>
<td>pT7-7</td>
<td>dehD', dehL', dehR' (deletion of promoter region)</td>
<td>This study</td>
</tr>
<tr>
<td>pHC773-ΔR</td>
<td>pT7-7</td>
<td>dehD', dehL', ΔdehR'</td>
<td>This study</td>
</tr>
<tr>
<td>pHC774</td>
<td>pT7-7</td>
<td>dehE', dehR'</td>
<td>This study</td>
</tr>
<tr>
<td>pFHXa/LIC1</td>
<td>pET-32</td>
<td>dehR'</td>
<td>This study</td>
</tr>
</tbody>
</table>
2.2 Growth conditions and media

Luria broth (LB) media was described by Miller (1972) and contained yeast extract 10.0 g/l, tryptone 5.0 g/l and NaCl 10.0 g/l.

PJC chloride-free minimal media was prepared as 10x concentrated basal salts containing K₂HPO₄ 42.5 g/l, NaH₂PO₄ 2H₂O (10.0 g/l) and (NH₄)₂SO₄ (25.0 g/l). The trace metal salts solution was a 10x concentrate that contained nitriloacetic acid (NTA) (1.0 g/l), MgSO₄ (2.0 g/l), FeSO₄ 7H₂O (120.0 mg/l), MnSO₄ 4H₂O (30.0 mg/l), ZnSO₄ H₂O (30 mg/l) and CoCl₂ (10.0 mg/l) in distilled water (Hareland et al., 1975).

Minimal media for growing bacteria contained 10ml of 10x basal salts and 10ml of 10x trace metal salts per 100ml of distilled water and were autoclaved (121°C, for 15 minutes). Carbon sources were sterilised separately and added aseptically to the media to the desired final concentration. Liquid minimal cultures were supplemented with yeast extract to a final concentration of 0.05% (w/v) for growth of *Rhizobium*.

Minimal medium M63 for the growth of *E. coli* strains was prepared as 5x concentrated stock containing K₂HPO₄ 136 g/l, [NH₄]₂SO₄ 20 g/l, FeSO₄ 7H₂O 5 mg/l and vitamin B1 10 mg/l. pH was adjusted to pH 7 by addition of KOH. The medium was prepared for use by adding 20 ml of basal salts (5x concentration) and 1 ml of sterile MgSO₄ (0.1M) per 100 ml of distilled water and autoclaved as before.

Liquid cultures were incubated in a Gallenkamp orbital shaker at 200 rpm at the appropriate temperature. The liquid media was solidified by addition of Oxoid bacteriological agar No. 1 (1.5% w/v) prior to sterilisation.

2.3 Glycerol stocks

Glycerol stocks of organisms were prepared by adding 0.3 ml of sterile 50% glycerol to 0.7 ml of bacterial culture. The sample was then mixed thoroughly and frozen by standing in dry ice/IMS bath. Stocks were stored at -80°C.
2.4 Preparation of plasmid DNA

Plasmids were isolated using the alkaline lysis method as described by Sambrook et al., (1989). Cells were harvested (1.5 ml) from an overnight LB/amp culture by centrifugation in a benchtop MSE microcentaur microcentrifuge for 1 minute at 12,000 g. The supernatant was removed and the cells resuspended in 100 μl of GTE solution (25 mM Tris-HCl pH 8, 10 mM EDTA, 50mM glucose) and left at room temperature for 5 minutes. Cell lysis solution (0.2M NaOH, 1% SDS;200μl) was added and then mixed by inversion and left on ice for 5 minutes. Potassium acetate (3M with respect to potassium, 5M with respect to acetate ; 150μl) was then added. The tube was mixed before being incubated on ice for a further 5 minutes, then centrifuged for 5 minutes at 12,000 g. The supernatant was transferred to a fresh tube and the pellet was discarded. Phenol/chloroform (500 μl) was added and then mixed by vortexing for 1 minute. The phases were separated by centrifugation (2 minutes at 12,000 g) and the upper aqueous layer was transferred to a fresh tube. Then it was extracted with 500 μl of chloroform, by vortexing, and the phases were separated by centrifugation (2 minutes at 12,000 g). The upper aqueous phase was transferred to a fresh tube. The plasmid DNA was precipitated by the addition of 0.1 volume of 3M sodium acetate pH 5.2 and 2 volumes of ice cold absolute ethanol. The solutions were mixed by inversion and then incubated on ice for 5 minutes. The DNA was pelleted by centrifugation for 10 minutes at 12,000 g and then the pellet was rinsed with 70% ethanol. The pellet of DNA was dried under vacuum and then dissolved in TE (Tris-EDTA pH 7.5) buffer solution. The volume depending on how much DNA is obtained.

2.5 Preparation of chromosomal DNA

Chromosomal DNA was prepared according to the method of Wilson (1994). Cells were grown overnight with vigorous shaking in 500 ml PJC medium with an appropriate carbon source in a 2 litre flask to ensure good aeration. Cells were harvested, when growth reached A_{600nm} of about 0.7, by centrifugation for 30 minutes at 9,000g at room temperature.

The cells were resuspended in 20 ml of STE buffer (0.5M sucrose, 25 mM EDTA, 25 mM Tris-HCl pH 8) and then centrifuged for 15 minutes at 9,000 g. The cells were resuspended in 20 ml of STE buffer containing lysozyme at 5 mg/ml and incubated at
37°C for 30 minutes. SDS (2.8 ml of 10% w/v) and 15 μl of proteinase K (20 mg/ml) were added and the reaction was incubated for 20 minutes at 50°C and then 10 minutes at 65°C. After incubation, 3.6 ml of 5M NaCl was added followed by 3.0 ml of 10% cetyl trimethylammonium bromide (CTAB) in 0.7M NaCl prewarmed to 65°C. The reaction was well mixed and then incubated at 65°C for 10 minutes. An equal volume of chloroform/Isoamylalcohol (24:1) was added and then the tubes were mixed to give a homogeneous solution. The phases were then separated by centrifugation for 10 minutes at 9,000g. The upper aqueous phase was extracted with an equal volume of phenol/chloroform/Isoamylalcohol (25:2:1). The reaction was mixed and then centrifuged for 15 minutes at 12,000g. The upper aqueous phase was removed and an equal volume of chloroform/Isoamylalcohol added. The solution was again mixed and then centrifuged for 10 minutes at 9,000g. The upper aqueous phase was removed and 0.6 volumes of ice cold isopropanol was added and then gently mixed. The precipitated DNA was spooled onto a sealed Pasteur pipette heated to give a hook. The DNA was washed in ice cold 70% ethanol, air dried and dissolved in an appropriate volume of distilled water overnight at 4°C. The DNA obtained was suitable for restriction digestion and Southern blotting.

2.6 Measurement of DNA concentration
DNA concentration was estimated by ultraviolet spectrophotometry. An A$_{260\text{nm}}$ of 1.0 corresponds to 50μg of double stranded DNA per ml. The DNA purity was estimated from the A$_{260\text{nm}}$/A$_{280\text{nm}}$ = ratio. Ratios of less than 1.8 indicate that the preparation is contaminated with protein (Maniatis et al., 1982).

2.7 Restriction enzyme digestion
Digestions with restriction endonucleases were carried out according to the instructions of the manufacturer using the buffer provided. Enzymes were purchased from Pharmacia Biotech. Routine digests of plasmids included 500 ng - 1μg of DNA in a total reaction volume of 15μl. Digestion times at 37°C ranged from 1-2 hours for plasmids to overnight for complete digestion of chromosomal DNA.
2.8 Phosphatase treatment of DNA
Calf intestinal phosphatase (CIP) was used to remove terminal phosphate groups. One unit of phosphatase was added to the digested DNA and incubated at 37°C for 30 minutes in the buffer recommended by the manufacturer. The reaction was terminated by heat inactivation at 85°C for 15 minutes.

2.9 Agarose gel electrophoresis
Restriction enzyme digests were analysed by submarine gel electrophoresis through agarose gels. Generally, 0.8% agarose gel prepared in TAE buffer (40 mM Tris-acetate pH 7.6, 1 mM EDTA + ethidium bromide at 0.5 μg/ml) was used. Samples (between 2 to 5 μl) were mixed with 5μl of gel loading buffer [0.25% bromophenol blue (w/v), 0.25% xylene cyanol (w/v), 30% glycerol (w/v)] before loading. Gels were run at a constant 90 volts for 1 to 2 hours (Sambrook et al., 1989).

DNA fragments on the gel were visualised using a UV transilluminator. The sizes were estimated by comparison with a 1 kb ladder (Life Technologies Inc.) standard DNA marker (1μg of DNA marker was used each time). The DNA Ladder could also be used as a means of estimating the amount of DNA present in a sample as the band at 1636 base pairs makes up 10 % (100 ng) of the total DNA present in the marker used.

2.10 Isolation of DNA fragments from agarose gels
Fragments generated from restriction digests were extracted from agarose gel using the JETsorb DNA extraction kit (Genomed Inc.) The desired fragments were identified by illumination with long wavelength UV light and corresponding regions of the gel were excised with a sterile scalpel blade and placed into a 1.5ml eppendorf tube. The DNA purification was carried out according to the instructions of the manufacturer.

2.11 Ligation of DNA
The ligation reactions contained vector and insert at a ratio of 1:4 and one unit of T4 DNA ligase using the supplied buffer. The reactions were carried out in a total volume of 10μl or 20μl and incubated at 16°C for 2 hours. Before use in transformation, the ligated DNA was diluted by taking 2μl from the reaction mixture into 23μl of sterile distilled water. During transformation all of the 25μl was used.
2.12 Polymerase Chain Reaction (PCR) for Amplification of DNA

PCR reactions were generally carried out in 50μl reaction. The components in the PCR reactions are:

- 10x buffer with MgSO$_4$ (Promega) 5.0μl
- dNTPs mix (10mM) 4.0μl
- Forward primer (20pmol/μl) 1.0μl
- Reverse primer (20pmol/μl) 1.0μl
- DNA template variable (e.g. >0.5μg)
- Pfu DNA polymerase (3u/μl) (Promega) 0.3μl
- Sterile distilled water to final volume of 50μl

The amplification was carried out using a Techne Progene thermal cycler. The program used was as follows:

<p>| | | | |</p>
<table>
<thead>
<tr>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation:</td>
<td>95°C</td>
<td>60 sec</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Denaturation:</td>
<td>95°C</td>
<td>1.5mins</td>
<td></td>
</tr>
<tr>
<td>Annealing:</td>
<td>between 50 to 55°C</td>
<td>40 sec</td>
<td>25 cycles</td>
</tr>
<tr>
<td>Extension:</td>
<td>72°C</td>
<td>1 mins</td>
<td></td>
</tr>
<tr>
<td>Final extension:</td>
<td>72°C</td>
<td>10 mins</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Stop:</td>
<td>4°C</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The exact parameters were dependent on a number of factors including estimated length of the final product and predicted annealing temperature of the primers. After completion the reaction mixture was electrophoresed on 0.8% agarose gel.
The primer concentration was calculated using the equation:

\[
\text{Concentration(pmol/\mu l)} = 100 \times \text{Absorbance}_{260nm} \times \text{dilution factor of the DNA solution} / A
\]

Where \( A = (nA \times 1.54) + (nG \times 1.17) + (nT \times 0.92) + (nC \times 0.75) \); \( n = \) is the number of that base in the oligodeoxyribonucleotide

2.13 Engineering of the \textit{dehR} gene into the pT7-7 vector

Plasmid pFH772 was constructed by insertion of a PCR fragment containing the \textit{dehR} gene into a pT7-7 vector. To incorporate the \textit{Ndel} site into the PCR product, the primer for the start (5'end) of the gene was designed (Table 2.3). The other primer at the 3’end with \textit{SalI} site incorporated into it was designed to ensure that the whole of the gene would be copied by the PCR (Table 2.3).

\begin{table}[h]
\centering
\begin{tabular}{|l|}
\hline
Table 2.3. PCR primers enabling the insertion of an \textit{Ndel} site at the start of the dehalogenase regulator gene and \textit{SalI} site at the end of the regulator gene  \\
\hline
\hline
PCR primer with an \textit{Ndel} site – that includes the atg initiation codon (in bold) at the start of the gene  \\
\hline
\texttt{5' g gaa ttc cat atg a a g a t g c t g c 3'}  \\
\hline
PCR primer to include \textit{SalI} site (in bold) after the end of the \textit{dehR} gene  \\
\hline
\texttt{5' a t t a c g t a g e c g e c c t c a t g c 3'}  \\
\hline
\end{tabular}
\end{table}
2.14 Engineering of the dehR gene into the pUC18 vector

The primer for the start of the gene was designed as shown in Table 2.4 with a BamHI site and a termination codon to prevent fusion protein formation. The primer used from the 3'end was the same primer as in Table 2.3.

Table 2.4. PCR primer enabling the insertion of a BamHI site upstream of the dehR gene

<table>
<thead>
<tr>
<th>PCR primer to include</th>
<th>5’ cgg gat cct tga ccg tcc ggt gcc tgg gc 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>BamHI site is underlined.</td>
<td></td>
</tr>
<tr>
<td>Stop codon incorporated is in bold</td>
<td></td>
</tr>
</tbody>
</table>

2.15 Engineering of the dehR gene into the pET-32 vector system

The procedures of ligating the dehR gene into the pET vector system were based on the commercially available NOVAGEN Xa/LIC vector system manual. Primers were designed to include sequences to generate vector compatible overhangs as shown in Figure 2.2 (a)(b). These primers were used in the PCR with pFH772 (dehR') as template DNA.

2.16 Preparation of competent cells and transformation of plasmid DNA

Competent E.coli cells were prepared by the method of Kushner (1978). The required strain was grown overnight in 5 ml LB cultures. The next day 100 µl of the overnight culture was subcultured into fresh LB and grown at 37°C to A680 nm of approximately 0.4. Aliquots of cells (1.5 ml) were harvested by centrifugation for 30 seconds at 12,000g and the drained pellets resuspended in 0.5 ml of sterile solution A (10 mM MOPS pH 7, 10 mM RbCl). The cells were then pelleted with a further centrifugation for 20 seconds at the same speed and resuspended in 0.5 ml of solution B (100 mM MOPS pH 6.5, 10 mM RbCl, 500 mM CaCl2) and left on ice for 60 - 90 minutes. Cells then were collected by a 10 second spin at the same speed and resuspended in 150 µl of solution B with 0.2% v/v dimethyl sulphoxide. DNA was added (10-200 ng) and the mixture left on ice for 1 hour. The cells were heat-shocked at 55°C for 30 seconds and chilled on ice for 1 minute.
Figure 2.2. (a) Overview of the ligation independent cloning (LIC) strategy for Xa/LIC vector.

The gene of interest is PCR-amplified using primers with 5' extensions to introduce complementary overhangs to the LIC vector after treatment with T4 DNA polymerase. The insert and vector are then annealed and transformed into competent *E.coli* cells where the backbone is ligated *in vivo*.

Sense primer: 5’ ggt att gag ggt cgc atg aaa ggg aat gac 3’
Antisense primer: 5’ aga gga gag tta gag cct cag gtc caa tcc gc 3’

Figure 2.2. (b) Schematic representation of the pET-32 Ek/LIC or pET-32 Xa/LIC vector products.

The LIC-vector encodes the thioredoxin protein of *E.coli* (Trx-tag), a hexa-histidine tag (His-tag), and the 15 amino acid S-tag. The gene of interest is inserted at the LIC-site. The thrombin and Ek/Factor Xa cleavage sites are shown.
Figure 2.2(a).

```
5'GGTATTGAGGGTCGC (or any) ATG  ---- TGA
TARGET GENE
TAC

CCGAGATTGAGAGGAGA

5'GGTATTGAGGGTCGCATG
TARGET GENE
TGAGGCTCTAACTCTCCTCT3'

3'CCATAACTCCAGCGTAC
CCGAGATTGAGAGGAGA5'

T4DNA pol + dGTP only

5'GGTATTGAGGGTCCGATG
TARGET GENE
TGAGG

CCGAGATTGAGAGGAGA

Insert

TCC
AGGCCATAACTCCCA

PLASMID
CTCTAACTCTCCTCT

Xa

annealing & transformation

5'TCCGGTATTGAGGGTCGCATG
TARGET GENE
TGAGGCTCTAACTCTCCTCT

AGGCCATAACTCCAGCGTAC
CCGAGATTGAGAGGAGA

Recombinant plasmid
```

Figure 2.2(b).

```
Thrombin Xa/Ek-LIC site

T7promoter
Trx. Tag His.Tag S.Tag MCS His.Tag
```
Then 1 ml of prewarmed SOC (2% Bactotryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl₂, 10mM MgSO₄, 20mM glucose) medium was added. The cells were then allowed to recover at 37°C for 1 hour then spread (100 μl) on a prewarmed LB/amp/IPTG/X-gal plate.

The LB/amp/IPTG/X-gal plate allowed blue/white screening. The presence of an insert DNA disrupts the α fragment of the vector (lacZ') which prevents the formation of β-galactosidase. The presence of white colonies indicates disruption of the α-fragment.

2.17 DNA sequencing and oligodeoxyribonucleotide synthesis

Sequencing was performed by the Protein & Nucleic Acid Chemistry Laboratory (PNACL), University of Leicester. Initial sequencing of both strands was carried out using an Applied Biosystems AB1377 automated sequencer by employing standard pUC18 reverse and forward primers.

Sequences were extended by designing downstream primers based on the available determined sequence. These oligodeoxyribonucleotide primers were made with an Applied Biosystems Model 380B DNA synthesizer using cyanoethyl-phosphoramidite chemistry at PNACL, University of Leicester.
2.18 Southern blot analysis

2.18.1 Transfer of DNA to nitrocellulose membranes

The DNA was transferred from an agarose gel to a nitrocellulose membrane using a capillary transfer method (Southern, 1975). The gel was treated with depurinating solution (0.25M HCl) for 10 minutes at room temperature in order to cause breakage of the DNA through depurination. This treatment improves the efficiency of transfer of large fragments. The DNA was then denatured by incubation of the gel with a solution of 1.5 M NaCl, 0.5 M NaOH for 30 minutes with gentle agitation. The denaturing solution was then removed and replaced with neutralising solution (3M NaCl, 0.5M Tris-Cl pH 7.4). The gel was incubated for a further 30 minutes and then removed to a fresh container and rinsed with distilled water.

The gel was placed on a piece of Whatman 3MM paper and air bubbles between the paper and the gel were removed. The gel was surrounded but not covered by nescofilm. This served as a barrier to prevent liquid from flowing directly from the reservoir to the paper towels placed on top of the gel.

A piece of Hybond-N membrane which had been cut to the same size as the gel and wetted by immersion in distilled water followed by soaking in transfer buffer for at least 5 minutes was then carefully placed onto the surface of the gel. Air bubbles between the membrane and the gel were removed by the application of gentle pressure. Two layers of 3MM paper were placed on top of the membrane followed by a stack of paper towels. The set up will allow the flow of liquid buffer (20x SSC: 0.3M Trisodium citrate, 3M NaCl pH 7 adjusted with HCl) from the reservoir through the gel and the nitrocellulose filter, so that fragments of denatured DNA were eluted from the gel and deposited on the nitrocellulose filter. The transfer of DNA was allowed to proceed for 16 hours at room temperature.

After transfer, the blotting apparatus was disassembled and the membrane was removed from the agarose gel. The gel was examined under a UV transilluminator to check all the DNA had been transferred onto the membrane. The membrane was then air-dried and exposed for 30 seconds to short wavelength UV light to fix the DNA to the Hybond-N nitrocellulose membrane.
2.18.2 Hybridisation using non-radioactive probing method

The blots were prehybridized and hybridized using a method based on non-radioactive materials (AlkPhos Direct - Gene Images by Amersham Life Science).

2.18.3 Preparation of labelled probe

DNA to be labelled was diluted to a concentration of 10ng/μl using the water supplied. Diluted DNA (10μl) was placed in a microcentrifuge tube and denatured for 5 minutes in a vigorously boiling water bath. After 5 minutes, the DNA was immediately cooled on ice for 5 minutes. Then 10μl of reaction buffer was added to the cooled DNA and mixed, followed by 2μl of labelling reagent (containing alkaline phosphatase; 0.1%(w/v) sodium azide). Finally, 10μl of cross-linker solution (containing 4.7%(v/v) formaldehyde) was added and incubated for 30 minutes at 37°C. The labelled probe can be used immediately or kept on ice for up to 2 hours.

2.18.4 Pre-hybridisation/hybridisation

The blot was pre-hybridised in a hybridisation buffer containing 12%(w/v) urea plus final concentration of 0.5M NaCl and 4% (w/v) “Blocking Reagent” for at least 3 hours at 65°C in a hybridisation oven. Then the labelled probe was added to the buffer and hybridised overnight in a rotating hybridisation oven.

2.18.5 Post-hybridisation stringency washes

Primary wash buffer (2M urea, 0.1%SDS, 50mM sodium phosphate pH7.0, 150mM NaCl, 10mM MgCl₂, 0.2% blocking reagent) was pre-heated to 55°C before use. Then it was added to the blots and mixed for 10 minutes at 55°C with gentle agitation. This step was repeated twice. Finally the blot was added into a secondary buffer (50mM Tris base, 0.1M NaCl pH10.0, 10mM MgCl₂) and mixed for 5 minutes at room temperature. This step was also repeated twice as before.
2.18.6 Signal generation and detection

Secondary wash buffer was drained off from the filter, which was then placed (top side up) on a flat surface. Detection reagent CDP-Star or substrate (chemiluminescent detection of alkaline phosphatase reagent containing <1.5% (w/v) Disodium 2-chloro-5-(4 methoxyspiro [1,2-dioxetane-3,2'-(5'-chloro)-tricyclo [3,3,1,1]decan]-4yl)phenyl phosphate) was pipetted (30-40µl/cm²) onto the blots and left for 2-5 minutes. The excess of detection reagent was drained off and the blot wrapped with cling film. Finally, the blot with the DNA was placed topside up in the film cassette for detection. A sheet of X-Ray film was placed on top of the blots and exposed for 1 to 3 hours at room temperature. The film was then developed using a standard automatic developer.

2.19 Dot blot assay

The DNA sample was denatured by heating at boiling temperature for five minutes. Then it was immediately chilled on ice for five minutes. The specimen was spotted onto a small area of nitrocellulose membrane (2-3 µg DNA per spot). The dot blot was air dried before fixing by UV irradiation. Finally, it was hybridised with non-radioactive labelled probe as described above.

2.20 Colony hybridisation (Grunstein and Hogness, 1975)

Colonies to be screened were patched and duplicated onto selection plates. After incubation overnight, one of each duplicate was placed at 4°C for storage whilst the other was used for screening. Nitrocellulose filters were then placed on the surface of the plate and left for 2-5 minutes (aligning marks were made) to transfer the colonies. The filters were carefully lifted from the agar surface and placed on 3MM Whatman paper pre-dampened with 10% SDS. After 5 minutes the filters were placed on a 0.5M NaOH pad for a further 5 minutes. After the alkali treatment the filters were neutralised using neutralisation solution (1.0M Tris-HCl, pH 7.5, 1.5M NaCl solution). The filters were then exposed to short wavelength UV light to crosslink the DNA onto the nitrocellulose membrane. Then the filters were hybridised using the same technique as in non-radioactive probing method.
2.21 Preparation of cell-free extracts

Cell-free extracts were prepared from bacterial cells in mid- to late-exponential phase of growth. Cells from 100ml culture were harvested by centrifugation at 10,000g for 10 minutes at 4°C. The cell pellets were resuspended in 20ml of 0.1M Tris-acetate buffer pH7.6 and centrifuged at 10,000g for 10 minutes at 4°C. The cells were then resuspended in 4ml of 0.1M Tris-acetate buffer pH7.6 and maintained at 0°C for ultrasonication in an MSE Soniprep 150W ultrasonic disintegrator at a peak amplitude λ=10 microns for 30 seconds. Sonication of *Rhizobium* cell suspensions was generally carried out for 3x30 second periods, with 30 seconds cooling between each sonication. Unbroken cells and cell wall material were removed by centrifugation at 20,000g for 15 minutes at 4°C. Cell-free extracts were ultracentrifuged at 120,000g for 90 minutes at 4°C for use in protein purification.

2.22 Preparation of DehE, DehL and DehD

2.22.1 Preparation of cell-free extracts containing DehE

*E.coli* strain BL21 (DE3) carrying plasmid (pJS771) containing the *dehE* gene were grown aerobically in a 100ml LB/amp overnight at 30°C. Then 15 to 20ml of the culture (\(A_{680nm}=1.60\)) was transferred into 500ml LB/amp in a 2L flask. This was incubated on an orbital shaker at 30°C. Absorbance was checked periodically until \(A_{680nm} = 0.3 - 0.4\) when 50μl of 0.1M IPTG was added to give the final concentration of 0.01mM. The cell culture was then incubated at 20°C overnight before harvesting by centrifugation at 10,000g for 15 minutes at 4°C.

The cell pellets were washed in 20ml 0.1M Tris-acetate buffer pH 7.6 and centrifuged as before to recover the pellet. The supernatant was discarded and the pellet was kept at -20°C until use. The extract was prepared as described before.

2.22.2 Preparation of cell-free extracts containing DehL and DehD

*E.coli* K-12 strain NM522 carrying plasmid pSC3 or pSC4 containing *dehD* and *dehL* gene, respectively, were grown in a 10ml LB/amp culture overnight at 37°C. Then cells from 2ml of the culture were washed with sterile PJC before inoculating into 20mM D,L2-CP minimal medium containing 0.05% yeast extract plus 0.3mM IPTG.
Cells were grown at 30°C and harvested at late logarithmic phase ($A_{680nm} 0.7$ to $0.8$). Cells were harvested and extract prepared as already described.

2.23 Preparation of inclusion bodies
The cells from 100 ml culture *E. coli* BL21(DE3)[pFH772] ($dehR^+$) were resuspended in 4ml of 0.1M Tris-acetate pH7.6 buffer followed by ultrasonication. The inclusion bodies were sedimented by centrifugation at 15,000g for 15 min at 4°C. The pellet then resuspended again in 0.1M Tris-acetate pH7.6 buffer solution. An appropriate amount of suspended pellet (0.8mg protein) was then added to 0.1M Tris-acetate pH7.6 buffer plus 8M urea and left at room temperature (21°C) for one hour. Insoluble material was removed by centrifugation at 15,000g for 15 minutes. This is important to remove existing aggregates that can act to trigger aggregation during folding.

2.24 Estimation of protein concentration
The protein concentration of crude cell extracts was measured using the biuret method described by Gornall *et al.* (1949). A standard curve of protein concentration was constructed using bovine serum albumin as reference. Cell-free extract (200 μl) was added to the biuret reagent (800 μl) mixed and the colour was allowed to develop for 30 minutes at room temperature before measuring at $A_{540nm}$ against a blank made using 200μl buffer. An $A_{540nm}$ value of 0.1 corresponds to a protein concentration of 2 mg/ml. The concentration of protein in column fractions was estimated by measurement of the $A_{280nm}$ (Warburg and Christian, 1941).

2.25 Assay for dehalogenase activity
Dehalogenase activity was determined as total chloride released at 30°C in an incubation mixture containing:

1- 0.1M Tris-acetate buffer(pH7.6)  4700μl
2- 0.1M halogenated aliphatic acid  50μl
3- Distilled water and enzyme
   to a final volume of  5000μl
After 5 minutes equilibration at 30°C, the reaction was initiated by adding cell-free extract. Samples (1.0ml) were removed at appropriate intervals and assayed for halide ions.

2.26 Assay for halide ion
Measurement of free halide released during the dehalogenation reaction was carried out by an adaptation of the method of Bergman and Sanik (1957).

Sample (1ml) was added into 100 μl of 0.25M ammonium ferric sulphate in 9M nitric acid and mixed thoroughly. To this was added 100μl mercuric thiocyanate-saturated ethanol and the solution was mixed by vortexing. The colour was allowed to develop for 10 minutes and measured at A460nm in a Pye-Unicam SP1750 Series spectrophotometer. Halide concentration was determined by comparison of the absorbance of the test sample against a standard curve of known concentrations of halide.

2.27 Standard curve for chloride and bromide ions
A standard curve for chloride ion was constructed using sodium chloride within the range of 0-0.2μmol. A standard curve for bromide ion (Br⁻) using sodium bromide was also constructed within the range of 0 -1.0 μmol Br⁻. Standards were known concentrations of sodium chloride and sodium bromide in 100mM Tris-acetate buffer pH 7.6 with colour developed as described in section 2.26. Both standard curves are shown in Figure 2.3 (a) and (b). Chemicals used were from Sigma with Analar grade.
Figure 2.3. (a) Standard curve of Cl$^-$ concentration (0 - 0.2$\mu$molCl$^-$) against $A_{460\text{nm}}$

Figure 2.3. (b) Standard curve of Br$^-$ concentration (0 - 1.0$\mu$molBr$^-$) against $A_{460\text{nm}}$
2.28 Techniques for protein analysis

2.28.1 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Methods are based on the protocol of Laemmli (1970)

2.28.1.1 Standard SDS-PAGE mini-gel preparation

SDS-PAGE mini-gels were prepared using the Mini-Protean II kit (Biorad). The composition formulation for mini-gel was described in Table 2.5.

Table 2.5. Preparation of acrylamide gel for SDS-PAGE

<table>
<thead>
<tr>
<th></th>
<th>Resolving gel</th>
<th>Stacking gel</th>
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<tbody>
<tr>
<td></td>
<td>12% acrylamide</td>
<td>4% acrylamide</td>
</tr>
<tr>
<td>Distilled water</td>
<td>4.35ml</td>
<td>3.15ml</td>
</tr>
<tr>
<td>1.5M Tris.Cl pH 8.8</td>
<td>2.50ml</td>
<td>-</td>
</tr>
<tr>
<td>0.5M Tris.Cl pH 6.8</td>
<td>-</td>
<td>1.26ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>100μl</td>
<td>50μl</td>
</tr>
<tr>
<td>40% Acrylamide/bisacrylamide</td>
<td>3.00ml</td>
<td>0.50ml</td>
</tr>
<tr>
<td>10% (NH₄)₂S₂O₈</td>
<td>50μl</td>
<td>25μl</td>
</tr>
<tr>
<td>Tetramethyl-ethylenediamine (TEMED)</td>
<td>10μl</td>
<td>10μl</td>
</tr>
</tbody>
</table>
Glass plates and spacers were assembled in the casting system and the separating gel was poured, covered with a layer of water saturated with butanol (use the top layer), and allowed to set for one hour. The overlay solution was then removed and the stacking gel was poured, a comb was inserted without creating air bubbles, to form the sample wells and the gel was allowed to set for 30 minutes.

The mini-gel was placed in an electrophoresis tank and the electrodes were covered with 1x Tris-glycine running buffer solution (5x stock: 1.5%[w/v] Tris base, 7.2%[w/v] glycine and 0.5%[w/v] SDS). Samples were prepared by heating an appropriate amount of protein for 3 minutes at 100°C with 0.3 volume of sample buffer (50mM Tris-HCl, pH6.8, 10%[w/v]glycerol, 2%[w/v] SDS, 5%[v/v]2-β-mercaptoethanol, 0.05%[w/v]bromophenol blue).

Gels were generally run for two hours at a constant voltage of 150V. The protein bands were stained with Coomassie blue R250 solution (comprising 0.5%[w/v] solution Coomassie blue R250 in 45% [v/v] methanol; 10%acetic acid) for 2-3 hours. Gels were then destained in a solution of 7.5% [v/v] acetic acid; 5%[v/v] methanol for 3-16 hours.

Gels were calibrated using SDS-VII protein markers from Sigma (Table 2.6) or Standard Mark12 Wide Range Protein Standards from Invitrogen (Figure 2.4).

Table 2.6. Molecular weight markers used for SDS-PAGE

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular Mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin, Bovine</td>
<td>66,000</td>
</tr>
<tr>
<td>Albumin, Egg</td>
<td>45,000</td>
</tr>
<tr>
<td>Glyceraldehyde-3-P-Dehydrogenase</td>
<td>36,000</td>
</tr>
<tr>
<td>Carbonic Anhydrase, Bovine</td>
<td>29,000</td>
</tr>
<tr>
<td>Trypsinogen, Bovine Pancreas</td>
<td>24,000</td>
</tr>
<tr>
<td>Trypsin Inhibitor, Soybean</td>
<td>20,000</td>
</tr>
<tr>
<td>α-Lactalbumin, Bovine Milk</td>
<td>14,200</td>
</tr>
</tbody>
</table>
Figure 2.4. Mark12 Wide Range Protein Standards from Invitrogen
2.28.1.2 Non-denaturing Polyacrylamide Gel Electrophoresis

In non-denaturing PAGE, the enzyme extract remains active. Gels were prepared based on the method of Hardman and Slater (1981(a)). Resolving gels containing 12% bis-Acrylamide in 375mM Tris.SO₄ pH 8.8 were polymerized by the addition of 0.05% ammonium persulphate and 0.05%TEMED. Stacking gels were formed from 4% bis-Acrylamide in 125mM Tris.SO₄ pH 6.8.

Gels were left overnight at 4°C before being used to allow the ammonium persulphate to decompose completely. Gels were run using a Mini-Protean II gel system from Biorad in 25mM Tris, 19mM glycine buffer (pH 8.3) at a constant voltage of 200V and a temperature 4°C for 1 hour. Samples were prepared as for cell extracts and mixed with 0.1 volumes of sample buffer (0.1% bromophenol blue; 10% glycerol; 100mM DTT in 50mM Tris-acetate pH 6.8). The gel was run until the dye front reached the bottom of the gel. Gels were then stained for dehalogenase activity.

2.28.1.3 Dehalogenase activity staining

The gel was incubated in 50mM Cl⁻ free halogenated substrate for 15 minutes at 30°C. Substrate was carefully removed and the gel was then placed in a 0.1M AgNO₃ solution and incubated in the dark until bands appeared on the gel due to the precipitation of AgCl. The gel was then washed with distilled water to remove the AgNO₃ and fixed by washing in 5% acetic acid for 10 minutes. The gel was finally washed with distilled water and stored in the dark and photographed as required.

2.29 Protein purification

2.29.1 Fast protein liquid chromatography (FPLC)

FPLC was carried out using a Pharmacia system with MonoQ HR 5/5 (anion exchange chromatography) and Superose 12 HR 10/30 (size exclusion chromatography) columns. Purifications were performed at 4°C in a cold room. For molecular weight determination, two Superose 12 columns were used in series for greater resolution.
2.29.2 Anion exchange chromatography
Anion exchange chromatography was performed using a linearly increasing gradient of phosphate ions. For the FPLC system two buffers are required, one containing low salt concentration (Buffer A) and the other containing high salt concentration (Buffer B). Depending on the dehalogenase buffer A contained 5 or 20mM Phosphate buffer pH 8, 1mM EDTA, 10%Glycerol, 1mM DTT and buffer B contained 100 or 200mM Phosphate buffer, 1mM EDTA, 10%Glycerol, 1mM DTT. These buffers are then mixed via a gradient controller to give a linear gradient of 5mM to 100mM or 20mM to 200mM of phosphate. Samples for purification were prepared as cell-free extracts and approximately 5mg of protein were applied to the MonoQ column per run. The column was run at a flow rate of 1ml min$^{-1}$. Fractions (usually 1ml) were collected and then assayed to determine which contained maximal dehalogenase activity. Active fractions were stored in ice until use.

2.29.3 Gel filtration
Gel filtration was carried out using two columns of Superose 12 connected in series. Columns were equilibrated overnight using a buffer containing 20mM Tris-acetate, 0.1M sodium acetate pH 7.6. The sodium acetate is used to increase the ionic strength and so stop any interaction with the gel matrix. Samples (0.2ml) from the MonoQ step were applied to the gel filtration column at approximately 0.5mg of protein per run. The column was run at a flow rate of 0.4 ml/min. For molecular weight determination the columns were calibrated using molecular weight standards from SIGMA, the relative molecular weights of these being: β-amylase (200 kDa), alcohol dehydrogenase (150kDa), bovine serum albumin (66kDa), carbonic anhydrase (29kDa) and cytochrome c (12.4kDa).

2.29.4 Dialysis of a purified dehalogenase enzyme
Active fractions (1-2ml) containing dehalogenase enzyme were pooled and dialysed against 200ml buffer containing 0.1M Tris-acetate, 1mM DTT, 1mM EDTA, 10% glycerol pH 7.6 at 4°C overnight to remove the phosphate buffer.
2.30 **Purification of D,L2-chloropropionic acid (D,L2-CP)**

A 1M solution of D,L-2-chloropropionic acid (Fluka Chemicals) was adjusted to a pH of approximately 1.0 by addition of concentrated nitric acid. The solution was then extracted three times with an equal volume of ethyl acetate and the organic phases were pooled and dried over anhydrous sodium sulphate. The resulting solution was then evaporated at 45°C in a rotary evaporator. The residue was redissolved in distilled water and neutralised with 10M NaOH before being made up to the original volume.

2.31 **Purification of 2,2-dichloropropionic acid (2,2DCP)**

A commercially available 2,2DCP was purified by recrystallisation. 2,2DCP was dissolved in distilled water (1g/ml) at 50°C, cooled and kept at 0°C for up to 3 hours. The resulting crystals were vacuum-filtered using Whatman filter paper no. 1 and dried at 30°C until no further reduction in weight occurred. The recrystallisation was repeated using the dried crystals to produce a twice recrystallised product.

2.32 **Computer Analysis**

Sequence analysis was carried out using the DNA Strider programme. Sequence alignment using the Genetics Computer Group (GCG) package was used to identify regions of similarity between two sequences.

International databases were searched using the BLAST programme (Altschul *et al.*, 1990) (http://www.ncbi.nlm.nih.gov). The 16S rRNA gene sequences was compared with those in the EMBL database (Guenter *et al.*, 1998) using FASTA3 at the European Bioinformatics Institute (http://www.ebi.ac.uk) and with those from the Ribosomal Data base Project by using SIMILARITY RANK to identify closely related sequences (Maidak, *et al.*, 1997).
CHAPTER 3

PARTIAL CHARACTERISATION OF THE RHIZOBIAL DEHALOGENASE ENZYMES
3.1 Introduction

*Rhizobium sp.* was originally selected from soil by enrichment with 2,2DCP (Dalapon) (Berry *et al.*, 1976). It could also utilise D,L2-CP as sole source of carbon and energy and was shown to make three inducible dehalogenases (Leigh, 1986). These enzymes differed in their substrate specificities. All three enzymes acted on D,L2-CP with DehL specific for L2-CP, DehD specific for D2-CP and DehE acting on both enantiomers. DehD and DehL between them acted on MCA, DCA, and D,L2,3-DCP with DehE itself acting on all of these compounds as well as on TCA. It is not clear why *Rhizobium sp.* makes DehL and DehD when DehE on its own could act on all the identified substrates of the other two dehalogenases and only DehE could utilise 2,2DCP, on which the organism was isolated. An investigation of the kinetic properties of each dehalogenase may shed light on this question.

The kinetic properties of the dehalogenases have not properly been characterised to date. An earlier investigation on Km values was carried out by Allison *et al.*, (1983) but the values appear to be surprisingly high in various cases and need to be studied further.

This chapter will describe ways of preparing the three dehalogenases for characterising them kinetically. Expression of the cloned dehalogenase genes in *E.coli* allowed the production of crude extracts that had the individual dehalogenase proteins free from other dehalogenases and so could be used in an initial characterisation of the enzymes without any purification.

3.2 Source of DehL, DehD and DehE enzymes

The genes encoding DehL and DehD were originally isolated from *Rhizobium sp.* chromosomal DNA as plasmid pSC2. Further subcloning of pSC2 into pUC18 resulted in pSC4 and pSC3, which expressed DehL and DehD, respectively (Cairns *et al.*, 1996). The *dehE* gene from *Rhizobium sp.* has been cloned and overexpressed in a high expression vector pT7-7 to enable large amounts of the enzyme to be produced (Stringfellow *et al.*, 1997). This construct is called pJS771.
3.2.1 Growth of *E. coli* K-12 strain NM522 carrying pSC4 (*dehL*) or pSC3 (*dehD*) on D,L2-CP minimal medium

*E. coli* NM522[pSC4] and *E. coli* NM522[pSC3] were inoculated into minimal medium flasks containing (i) 20mM D,L2-CP + 0.05% Yeast Extract and (ii) 20mM 2CP only. IPTG (final concentration 0.3mM) was added to the growth medium before incubating at 30°C. The A$_{680}$nm readings were taken every two hours. The growth curves and the maximum growth measured at A$_{680}$nm are shown in Figure 3.1(a) for NM522[pSC4] and Figure 3.1(b) for NM522[pSC3]. From these two curves, the doubling time was quicker in the presence of yeast extract (doubling time, Td = 3 hours) than without yeast extract (doubling time, Td = 8 hours).

Cells were harvested at late log phase and extracts were assayed for dehalogenase activity using L-2CP and D-2CP as substrate for DehL and DehD, respectively. The enzyme activity was somewhat higher in cell-free extract prepared from 20mM D,L2-CP + Yeast extract-grown cells (Table 3.1).

**Table 3.1. Specific activity for DehD and DehL using D-2CP and L-2CP as substrates**

(Values are a mean of triplicate determinations)

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>Specific activity (μmolCl/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>E. coli</em> NM522 [pSC3] (dehD&lt;sup&gt;+&lt;/sup&gt;)</td>
</tr>
<tr>
<td>(20mM D,L2-CP + 0.05%Yeast extract)</td>
<td>1.61</td>
</tr>
<tr>
<td>(20mM D,L2-CP only)</td>
<td>1.40</td>
</tr>
</tbody>
</table>
Figure 3.1(a). Growth of *E. coli* NM522 [pSC4] in D,L2-CP minimal medium

Figure 3.1(b). Growth of *E. coli* NM522 [pSC3] in D,L2-CP minimal medium
3.2.2 Growth of *E. coli* BL21(DE3) carrying pJS771(*dehE*<sup>+</sup>)

BL21(DE3) transformed with pJS771 were grown overnight in 10ml LB/amp at 37°C. Then 2ml of overnight culture was added into a fresh 100ml LB/amp flask and grown at 30°C. IPTG (final concentration of 0.01mM) was added when $A_{680nm}$ reached 0.4 and the cells incubated at 20°C overnight. Cells were harvested and extract was prepared and checked for DehE activity. The activity against 2,2DCP was 1.20μmolCl⁻/min/mg protein.

3.2.3 SDS-PAGE analysis of DehL, DehD and DehE

SDS-PAGE analysis of the crude cell-free extracts was carried out to show how much dehalogenase protein had been produced. The expected band at 31kDa for DehL was absent from the control lacking *dehL* gene. SDS-PAGE for DehD showed the expected 29kDa protein was expressed (Figure 3.2). The amount of DehL and DehD was estimated visually to be about 20 – 30% of the total cellular proteins indicating that only 5-fold purification would be needed to obtain the pure enzymes.

SDS-PAGE analysis of the DehE cell-free extract showed a strong band at 32kDa indicating that the protein is present principally in the soluble form (Figure 3.3). However, there was a slight problem using this vector with the formation of inclusion bodies leading to some insoluble DehE. Figure 3.3 also shows that the enzyme accounted for about 30% of the total cellular proteins as visually estimated. Therefore, approximately 3-fold purification is needed to obtain a homogenous protein.

3.3 Checking the stability of DehL, DehE and DehD in crude extract

This experiment was carried out to check the stability of each enzyme for use in further investigations. The stability of each crude preparation of dehalogenase in 0.1M Tris-acetate buffer (pH7.6) was examined over 10 days storage at ice temperature (0°C). Extracts were assayed for dehalogenase activity using a specific substrate of D-2CP and L-2CP for DehD and DehL, respectively, and 2,2DCP for DehE. The extracts were examined from the day they were prepared (day 0) to day 10.
Figure 3.2. SDS-PAGE analysis of crude extracts from *E.coli* NM522[pSC4] (*dehL*) and *E.coli* NM522[pSC3] (*dehD*) grown on D,L2-CP minimal medium

Lane 1: SDS VII marker proteins
Lane 2: crude extract from NM522[pSC4] (*dehL*) (8µg protein)
Lane 3: NM522[pUC18] (8µg protein)
Lane 4: crude extract from NM522[pSC3] (*dehD*) (8µg protein)
Lane 5: SDS VII marker proteins
Figure 3.3. SDS-PAGE analysis of the fractionated crude extract from *E.coli* BL21(DE3)[pJS771] (*dehE*<sup>+</sup>)

Lane 1: Supernatant (5 µg protein)
Lane 2: SDS VII marker proteins
Lane 3: Resuspended pellet (5 µg protein)
Figure 3.4. Stability of DehL, DehE and DehD in crude extracts from 0 to 10 days during storage at 0°C

As can be seen in Figure 3.4, the enzymes remain stable for 24 hours but then the activity of both DehL and DehD started to decrease. Storage at 0°C resulted in a 33% loss for DehL activity and 64% loss for DehD activity over 10 days. However, DehE, was found to be stable. Therefore, all measurements were made on the same day that the enzyme was prepared or within 24 hours of preparation.

3.4 Dehalogenase specificity

The previously proposed specificity of DehL and DehD by Leigh (1986) was re-investigated using crude extract preparation. DehL (100μg protein) was tested with D-2CP, MCA, 2,2DCP and TCA and no chloride release was detected in the assay. Likewise, the activity of DehD (100μg protein) was tested using L-2CP, DCA, 2,2DCP and TCA and again no chloride release was observed. However, DehE (10μg protein) reacted with all the substrates. The specific activities for DehL, DehE and DehD for their various substrates are shown in Table 3.2.

An analog of D,L2-CP, D,L2,3-DCP was also studied. The amount of D or L isomer in D,L2,3-DCP was assumed to be 50%, similar to the D,L2-CP. In the enzyme assay the DehE enzyme (100 μg protein) was used with 1mM D,L2,3-DCP. The assay resulted in 1mM chloride ion being released at the completion of the reaction. If all the chloride was released, 2mM should be present, but this was not the case. This
suggested that chloride from only one position was released. Allison (1981) reported that chloride was not released from 3CP so presumably the chloride was released from carbon 2. In further experiments, when either DehD or DehL enzyme was used with 1mM D,L2,3-DCP as substrate, only 0.5mM chloride ion was released at the completion of the reaction. This was consistent with the known stereospecificity of DehD and DehL on the racemic chloride of carbon number 2. Since it was believed that only the chloride at carbon position 2 was attacked, the putative product was 2-hydroxy-3-chloropropionic acid for DehE and the same putative product plus unreacted substrate, either D- or L2,3-DCP, when DehL or DehD enzyme was used.

### Table 3.2. Specific activity of crude Rhizobial dehalogenases with their various substrates

(Values are the means of triplicate determinations)

<table>
<thead>
<tr>
<th>Substrates</th>
<th>DehL</th>
<th>DehE</th>
<th>DehD</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,2DCP</td>
<td>-</td>
<td>1.15</td>
<td>-</td>
</tr>
<tr>
<td>L-2CP</td>
<td>5.00</td>
<td>3.24</td>
<td>-</td>
</tr>
<tr>
<td>D-2CP</td>
<td>-</td>
<td>1.41</td>
<td>1.52</td>
</tr>
<tr>
<td>MCA</td>
<td>-</td>
<td>1.00</td>
<td>0.40</td>
</tr>
<tr>
<td>DCA</td>
<td>1.82</td>
<td>0.35</td>
<td>-</td>
</tr>
<tr>
<td>TCA</td>
<td>-</td>
<td>0.08</td>
<td>-</td>
</tr>
</tbody>
</table>

(-) indicate: no reaction
3.5 Determination of \( \text{Km} \) values for dehalogenase enzymes with various substrates

A range of substrates was used to obtain \( \text{Km} \) values for DehL, DehE and DehD. The \( \text{Km} \) values were determined to see if there was any significant difference between the enzymes, which act on the same substrates. \( \text{Km} \) determinations were described in detail in Appendix I.

The overall results are shown in Table 3.3. Compounds that are not a substrate for an enzyme are indicated by a dash. Using DehD, the \( \text{Km} \) for D-2CP was 0.06mM, 13x lower than that given by DehE (0.85mM). The \( \text{Km} \) for L-2CP using DehL was 0.13mM, which is 4x lower than that given by the DehE enzyme (0.56mM). When D,L-2-CP with equimolar amounts of the two isomers was used the \( \text{Km} \) values were very similar to those with the single enantiomer. This indicates that under these conditions, the presence of the non-substrate enantiomer has little or no effect on \( \text{Km} \). The \( \text{Km} \) of DehE for 2,2DCP (a growth substrate for *Rhizobium sp.* ) was 0.12mM. The \( \text{Km} \) values for various chlorinated and brominated acetic acids are given in Table 3.4. The value of \( \text{Km} \) decreased as the number of chloride or bromide atoms increased.

From all this information on \( \text{Km} \) values, the actual substrate concentration to use for enzyme assay to ensure that the enzyme was saturated with substrate could be determined.

3.6 Preparation of pure dehalogenases for more detailed kinetic analysis

To determine the \( \text{Kcat} \) and the Specificity Constant values the pure dehalogenase protein is needed. In the following section, ways of preparing the pure enzymes from crude extracts will be described.
Table 3.3. Km values (mM) for different substrates using crude dehalogenase preparations (Values are the means of triplicate determinations)

<table>
<thead>
<tr>
<th>Substrates</th>
<th>DehL</th>
<th>DehE</th>
<th>DehD</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-2CP</td>
<td>-</td>
<td>0.85±0.09</td>
<td>0.07±0.01</td>
</tr>
<tr>
<td>L-2CP</td>
<td>0.13±0.04</td>
<td>0.56±0.09</td>
<td>-</td>
</tr>
<tr>
<td>D,L-2CP</td>
<td>0.16±0.02*</td>
<td>0.41±0.05</td>
<td>0.08±0.01*</td>
</tr>
<tr>
<td>2,2DCP</td>
<td>-</td>
<td>0.12±0.02</td>
<td>-</td>
</tr>
<tr>
<td>D,L-2,3DCP</td>
<td>0.02±0.01*</td>
<td>0.94±0.26</td>
<td>0.53±0.06*</td>
</tr>
<tr>
<td>MCA</td>
<td>-</td>
<td>1.88±0.99</td>
<td>1.02±0.34</td>
</tr>
<tr>
<td>DCA</td>
<td>0.13±0.04</td>
<td>0.51±0.11</td>
<td>-</td>
</tr>
<tr>
<td>TCA</td>
<td>-</td>
<td>0.31±0.06</td>
<td>-</td>
</tr>
<tr>
<td>MBA</td>
<td>-</td>
<td>2.37±0.35</td>
<td>0.62±0.11</td>
</tr>
<tr>
<td>DBA</td>
<td>0.19±0.07</td>
<td>0.89±0.21</td>
<td>-</td>
</tr>
<tr>
<td>TBA</td>
<td>-</td>
<td>0.22±0.06</td>
<td>-</td>
</tr>
</tbody>
</table>

Note: ( - ): not a substrate for enzyme
( * ): Km values corrected for L or D isomer

Table 3.4. Km values for halogenated acetic acids using DehE enzyme (Values are the means of triplicate determinations)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Km(mM)</th>
<th>Substrate</th>
<th>Km(mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCA</td>
<td>1.88±0.99</td>
<td>MBA</td>
<td>2.37±0.35</td>
</tr>
<tr>
<td>DCA</td>
<td>0.51±0.11</td>
<td>DBA</td>
<td>0.89±0.21</td>
</tr>
<tr>
<td>TCA</td>
<td>0.31±0.06</td>
<td>TBA</td>
<td>0.22±0.06</td>
</tr>
</tbody>
</table>
3.6.1 Purification of the DehE enzyme

Cell-free extract was prepared in 0.1M Tris-acetate buffer pH7.6. Approximately 6mg protein (6U enzyme with 2,2DCP as substrate) was loaded onto a MonoQ HR 5/5 anion-exchange column equilibrated with 20mM sodium phosphate, 1mM EDTA, 1mM dithiothreitol(DTT), 10%(mass/vol.) glycerol buffer, pH7.6 and eluted with a sodium phosphate gradient to 200mM. DehE was eluted in two fractions at approximately 80mM sodium phosphate. Each fraction had 2.7U and 2.9U enzyme and a specific activity of 2.1U/mg and 2.9U/mg with 2,2DCP as substrate respectively, with a recovery of 93% in total (Figure 3.5). Analysis of the fractions by SDS-PAGE showed that at least 95% of the protein seen in each fraction was accounted for by a 32kDa band (Figure 3.6). Based on the protein gel analysis the protein from this one step purification was sufficiently pure for further characterisation.

3.6.1.1 Determination of the native molecular weight of DehE

The purified protein was applied to two Superose 12 columns (Pharmacia) connected in series. These columns had previously been calibrated using various molecular weight markers (Materials and Methods, section 2.29.3). The elution volume of DehE was 26.2ml and the native molecular weight was calculated using the equation of the standard curve straight line (Figure 3.7). The native molecular weight was found to be 62kDa. From the subunit molecular weight of 32kDa, this suggests that the protein is a dimer, like DehL and DehD (Cairns, 1994).
Figure 3.5. MonoQ elution profile for DehE enzyme

Extract was loaded onto a MonoQ 5/5 column equilibrated as described in the text and eluted with a linear 20 to 200mM sodium phosphate gradient pH7.6. The elution profile of DehE in fractions 13 and 14 (2.7 U and 2.9U) respectively, is superimposed on the protein trace.
Figure 3.6. SDS-PAGE analysis of the purification of DehE

Lane 1: Protein markers
Lane 2: Crude extract of DehE
Lane 3: MonoQ fraction 12
Lane 4: MonoQ fraction 13 (6µg protein)
Lane 5: MonoQ fraction 14 (6µg protein)
Lane 6: MonoQ fraction 15
Figure 3.7. The calibration curve of the Superose 12 gel filtration column

\[ y = -0.158(x) + 5.933 \]

\( y = \log \text{molecular weight} \)

\( x = \text{elution volume} \)

Elution volume of DehE = 26.2 ml,

\[ y = -0.158(26.2 \text{ml}) + 5.933 \]

\( y = 1.79 \)

Molecular weight = 62.14 kDa
3.6.2 **Purification of the DehL enzyme**

Cell-free extract was prepared in 0.1M Tris-acetate buffer pH 7.6 as described before. Approximately 2.5mg protein (4U enzyme) was applied to a Mono Q HR 5/5 anion-exchange column equilibrated with 10mM sodium phosphate, 1mM EDTA, 1mM dithiothreitol (DTT), 10%(w/v) glycerol buffer, pH 7.6 and eluted with sodium phosphate gradient to 100mM. DehL eluted in one fraction at approximately 45mM sodium phosphate. The peak had 4U enzyme and a specific activity of 20.0U/mg with L-2CP as substrate with an estimated recovery of 100% (Figure 3.8). Analysis of the fraction by SDS-PAGE showed that at least 90% of the protein seen was accounted for by a 31kDa band (Figure 3.9).

3.6.3 **Purification of the DehD enzyme**

Cell-free extract was prepared in 0.01M Tris-acetate buffer pH 7.6 and 2.8mg protein (4.3U enzyme) was applied to a Mono Q HR 5/5 anion-exchange column equilibrated with 5mM sodium phosphate, 1mM EDTA, 1mM dithiothreitol (DTT), 10%(mass/vol.) glycerol buffer, pH 7.6 and eluted with sodium phosphate gradient to 100mM. DehD eluted in four fractions between approximately 30mM and 40mM sodium phosphate. Each fraction had 1.3U, 1.0U, 1.15U and 0.5U enzyme, respectively and the enzyme recovered was estimated to be 92% (Figure 3.10). Analysis of the fractions by SDS-PAGE is shown in Figure 3.11. In Fraction 11, there was slight contamination from host cellular protein. Specific activity of one of the fractions was measured (Fraction 10) which gave 14.4U/mg with D-2CP as substrate. Since more than one fraction had DehD, possibly there are different forms of DehD protein. The Km value for each fraction was determined using D-2CP as substrate. All showed similar Km values of approximately 0.06mM, suggesting that they are the same DehD protein. A possible reason for the protein being eluted at different ionic strengths is that a host protease may act on the dehalogenase protein. This problem may be avoided by expressing the gene in a protease-negative host cell.
Figure 3.8. MonoQ elution profile of DehL enzyme

Extract was loaded onto a MonoQ 5/5 column equilibrated as described in the text and eluted with a linear 10 to 100mM sodium phosphate gradient pH7.6. The elution profile of DehL in fraction 11 (4U) is superimposed on the protein trace.
Figure 3.9. SDS-PAGE analysis of the purification of DehL.

Lane 1: MonoQ fraction 10
Lane 2: MonoQ fraction 11 (8μg protein)
Lane 3: MonoQ fraction 12
Lane 4: MonoQ fraction 13
Lane 5: MonoQ fraction 14
Lane 6: Crude extract of DehL
Lane 7: Protein markers
Figure 3.10. MonoQ elution profile for DehD enzyme

Extract was loaded onto a MonoQ 5/5 column equilibrated as described in the text and eluted with a linear 5 to 100mM sodium phosphate gradient pH7.6. The elution profile of DehD in fractions 10, 11, 12, and 13 (1.3, 1.0, 1.15, 0.5U) respectively, is superimposed on the protein trace.
Figure 3.11. SDS-PAGE analysis of the purification of DehD

Lane 1: Protein markers.
Lane 2: MonoQ fraction 9
Lane 3: MonoQ fraction 10 (4µg protein)
Lane 4: MonoQ fraction 11 (4µg protein)
Lane 5: MonoQ fraction 12 (3µg protein)
Lane 6: MonoQ fraction 13 (1µg protein)
Lane 7: MonoQ fraction 14
Lane 8: Protein markers
Lane 9: Crude extract of DehD
3.6.4 Km/Vmax/Kcat/Specificity Constant determination for a range of substrates using pure dehalogenase enzymes

The overall results for Km determinations are shown in Table 3.5 (Appendix I) and Vmax values in Table 3.6. Compounds that are not a substrate are indicated by a dash as described earlier.

When Km values with pure enzyme and crude extract were compared, (Table 3.5 and Table 3.3) there are no major differences. This suggests that the total cellular protein of the host cell did not affect dehalogenase substrate binding properties.

The Km values for D or L and D,L isomers for both chlorinated and brominated compounds were compared (Table 3.5). D,L-2BP was included for Km determination because it was a substrate for growth of Rhizobium sp. (Allison, 1981). Km for D-2CP using DehD enzyme was 0.06mM, which was 9x lower than that for DehE (0.52mM). In contrast, the Km for D2-BP using DehD was 0.48mM, and a similar Km for the same substrate (0.46mM) was seen using DehE.

The Km for L-2CP with DehL was 0.15mM, which was approximately 3x lower than the Km of the same substrate using DehE (0.41mM). However, the Km for L-2BP with DehL enzyme was 0.11mM again approximately 3x lower than the Km for the same substrate using DehE (0.29mM). Results in Table 3.5 indicated that DehD has a high affinity towards D-2CP and D,L2-CP but D-2BP and D,L2-BP were significantly less good substrates. On the other hand, DehL showed equivalent Km values for L-2CP and L-2BP. Km for D,L2,3-DCP using DehL (0.03mM) was approximately 10x lower than using DehE (0.36mM) and DehD (0.38mM).

Table 3.4 in the previous section indicated that the Km for TBA was more or less similar to TCA. With pure DehE enzyme the Km for TBA again was equivalent to that for TCA. The Vmax values in Table 3.6 were determined to calculate the Kcat and the Specificity Constants for the three dehalogenases.
Table 3.5. Km values (mM) for different substrates using pure dehalogenases
(Values are the means of triplicate determinations)

<table>
<thead>
<tr>
<th>Halogenated Compound</th>
<th>DehL</th>
<th>DehE</th>
<th>DehD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Km values (mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-2CP</td>
<td>0.52±0.16</td>
<td>0.06±0.01</td>
<td></td>
</tr>
<tr>
<td>D-2BP</td>
<td>0.46±0.04</td>
<td>0.48±0.09</td>
<td></td>
</tr>
<tr>
<td>L-2CP</td>
<td>0.15±0.02</td>
<td>0.41±0.14</td>
<td>-</td>
</tr>
<tr>
<td>L-2BP</td>
<td>0.11±0.01</td>
<td>0.29±0.05</td>
<td>-</td>
</tr>
<tr>
<td>D,L-2CP</td>
<td>0.12±0.01*</td>
<td>0.35±0.03</td>
<td>0.04±0.01*</td>
</tr>
<tr>
<td>D,L-2BP</td>
<td>0.10±0.01*</td>
<td>0.22±0.04</td>
<td>0.40±0.04*</td>
</tr>
<tr>
<td>2,2-DCP</td>
<td>0.19±0.03</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D,L-2,3DCP</td>
<td>0.03±0.01*</td>
<td>0.36±0.01</td>
<td>0.38±0.11*</td>
</tr>
<tr>
<td>MCA</td>
<td>1.19±0.13</td>
<td>0.95±0.04</td>
<td></td>
</tr>
<tr>
<td>DCA</td>
<td>0.13±0.01</td>
<td>0.36±0.12</td>
<td>-</td>
</tr>
<tr>
<td>TCA</td>
<td>0.31±0.05</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MBA</td>
<td>2.18±0.30</td>
<td>0.67±0.17</td>
<td></td>
</tr>
<tr>
<td>DBA</td>
<td>0.27±0.09</td>
<td>0.88±0.14</td>
<td>-</td>
</tr>
<tr>
<td>TBA</td>
<td>0.32±0.05</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Note: ( - ): not a substrate for enzyme
( * ): Km values corrected for L or D isomer
Table 3.6. Vmax values for different substrates using pure dehalogenases

<table>
<thead>
<tr>
<th>Halogenated Compound</th>
<th>DehL</th>
<th>DehE</th>
<th>DehD</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-2CP</td>
<td>-</td>
<td>0.0079±0.0009</td>
<td>0.0078±0.0002</td>
</tr>
<tr>
<td>D-2BP</td>
<td>-</td>
<td>0.020±0.0004</td>
<td>0.194±0.044</td>
</tr>
<tr>
<td>L-2CP</td>
<td>0.020±0.00074</td>
<td>0.013±0.0014</td>
<td>-</td>
</tr>
<tr>
<td>L-2BP</td>
<td>0.020±0.00024</td>
<td>0.013±0.00058</td>
<td>-</td>
</tr>
<tr>
<td>D,L-2CP</td>
<td>0.023±0.00055</td>
<td>0.0097±0.00026</td>
<td>0.0065±0.0001</td>
</tr>
<tr>
<td>D,L-2BP</td>
<td>0.017±0.00037</td>
<td>0.012±0.00065</td>
<td>0.20±0.0045</td>
</tr>
<tr>
<td>D,L-2,3DCP</td>
<td>0.0033±0.00075</td>
<td>0.0014±0.00001</td>
<td>0.031±0.00393</td>
</tr>
<tr>
<td>2,2-DCP</td>
<td>-</td>
<td>0.0054±0.00019</td>
<td>-</td>
</tr>
<tr>
<td>MCA</td>
<td>-</td>
<td>0.025±0.00079</td>
<td>0.0044±0.00015</td>
</tr>
<tr>
<td>MBA</td>
<td>-</td>
<td>0.087±0.0034</td>
<td>0.38±0.032</td>
</tr>
<tr>
<td>DCA</td>
<td>0.0062±0.0002</td>
<td>0.0016±0.00015</td>
<td>-</td>
</tr>
<tr>
<td>DBA</td>
<td>0.085±0.00087</td>
<td>0.014±0.00064</td>
<td>-</td>
</tr>
<tr>
<td>TCA</td>
<td>-</td>
<td>0.0002±0.0000</td>
<td>-</td>
</tr>
<tr>
<td>TBA</td>
<td>-</td>
<td>0.002±0.00008</td>
<td>-</td>
</tr>
</tbody>
</table>

Note: ( - ) indicates not a substrate for enzyme
Km and Kcat are two kinetic parameters which are useful for comparing reaction mechanisms of the three enzymes and to evaluate the kinetic efficiency of the enzymes. The Kcat value is equivalent to the number of substrate molecules converted to product in a given unit of time by 1mole of enzyme. The best way to compare the catalytic efficiencies for different substrates by the same enzyme is to compare the ratio Kcat/Km. This parameter is known as the Specificity Constant and is given in Table 3.7(i) to 3.7(iii) for each dehalogenase.

Table 3.7(i). Kcat and Specificity Constants for DehL enzyme

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Kcat(sec⁻¹)</th>
<th>Km</th>
<th>Specificity Constant (M⁻¹sec⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-2CP</td>
<td>20.00</td>
<td>1.50 x 10⁻⁴M (0.15mM)</td>
<td>1.33 x 10⁵</td>
</tr>
<tr>
<td>L-2BP</td>
<td>20.00</td>
<td>1.10 x 10⁻⁴M (0.11mM)</td>
<td>1.81 x 10⁵</td>
</tr>
<tr>
<td>D,L-2CP</td>
<td>25.00*</td>
<td>1.20 x 10⁻⁴M (0.12mM)*</td>
<td>2.08 x 10⁵*</td>
</tr>
<tr>
<td>D,L-2BP</td>
<td>17.40*</td>
<td>1.00 x 10⁻⁴M (0.10mM)*</td>
<td>1.74 x 10⁵*</td>
</tr>
<tr>
<td>D,L-2,3-DCP</td>
<td>03.28*</td>
<td>3.00 x 10⁻⁵M (0.03mM)*</td>
<td>1.05 x 10⁵*</td>
</tr>
<tr>
<td>DCA</td>
<td>06.25</td>
<td>1.30 x 10⁻⁴M (0.13mM)</td>
<td>4.80 x 10⁴</td>
</tr>
<tr>
<td>DBA</td>
<td>81.16</td>
<td>2.70 x 10⁻³M (0.27mM)</td>
<td>3.04 x 10⁵</td>
</tr>
</tbody>
</table>

(*) : values corrected for L isomer
Table 3.7(ii). Kcat and Specificity Constants for DehE enzyme for all the substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Kcat (sec⁻¹)</th>
<th>Km</th>
<th>Specificity Constant (M⁻¹sec⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-2CP</td>
<td>08.16</td>
<td>5.20 x 10⁻⁴ M (0.52 mM)</td>
<td>1.56 x 10⁴</td>
</tr>
<tr>
<td>D-2BP</td>
<td>20.66</td>
<td>4.60 x 10⁻⁴ M (0.46 mM)</td>
<td>4.40 x 10⁴</td>
</tr>
<tr>
<td>L-2CP</td>
<td>13.43</td>
<td>4.10 x 10⁻⁴ M (0.41 mM)</td>
<td>3.27 x 10⁴</td>
</tr>
<tr>
<td>L-2BP</td>
<td>13.43</td>
<td>2.90 x 10⁻⁴ M (0.29 mM)</td>
<td>4.60 x 10⁴</td>
</tr>
<tr>
<td>D,L-2CP</td>
<td>10.03</td>
<td>3.50 x 10⁻⁴ M (0.35 mM)</td>
<td>2.86 x 10⁴</td>
</tr>
<tr>
<td>D,L-2BP</td>
<td>12.40</td>
<td>2.20 x 10⁻⁴ M (0.22 mM)</td>
<td>5.64 x 10⁴</td>
</tr>
<tr>
<td>2,2-DCP</td>
<td>05.58</td>
<td>1.90 x 10⁻⁴ M (0.19 mM)</td>
<td>2.94 x 10⁴</td>
</tr>
<tr>
<td>D,L-2,3DCP</td>
<td>01.44</td>
<td>3.60 x 10⁻⁴ M (0.36 mM)</td>
<td>0.40 x 10⁴</td>
</tr>
<tr>
<td>MCA</td>
<td>25.83</td>
<td>1.19 x 10⁻³ M (1.19 mM)</td>
<td>2.17 x 10⁴</td>
</tr>
<tr>
<td>DCA</td>
<td>01.65</td>
<td>3.60 x 10⁻⁴ M (0.36 mM)</td>
<td>0.46 x 10⁴</td>
</tr>
<tr>
<td>TCA</td>
<td>00.20</td>
<td>3.10 x 10⁻⁴ M (0.31 mM)</td>
<td>0.65 x 10⁴</td>
</tr>
<tr>
<td>MBA</td>
<td>89.90</td>
<td>2.18 x 10⁻³ M (2.18 mM)</td>
<td>4.12 x 10⁴</td>
</tr>
<tr>
<td>DBA</td>
<td>14.46</td>
<td>8.80 x 10⁻⁴ M (0.88 mM)</td>
<td>1.64 x 10⁴</td>
</tr>
<tr>
<td>TBA</td>
<td>02.06</td>
<td>3.20 x 10⁻⁴ M (0.32 mM)</td>
<td>0.64 x 10⁴</td>
</tr>
</tbody>
</table>

Table 3.7(iii). Kcat and Specificity Constants for DehD enzyme

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Kcat (sec⁻¹)</th>
<th>Km</th>
<th>Specificity Constant (M⁻¹sec⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-2CP</td>
<td>07.45</td>
<td>6.00 x 10⁻⁵ M (0.06 mM)</td>
<td>1.12 x 10⁵</td>
</tr>
<tr>
<td>D-2BP</td>
<td>187.53</td>
<td>4.80 x 10⁻⁴ M (0.48 mM)</td>
<td>3.90 x 10⁵</td>
</tr>
<tr>
<td>D,L-2CP</td>
<td>06.28*</td>
<td>4.00 x 10⁻⁵ M (0.04 mM)*</td>
<td>1.46 x 10⁵*</td>
</tr>
<tr>
<td>D,L-2BP</td>
<td>193.33*</td>
<td>4.00 x 10⁻⁵ M (0.40 mM)*</td>
<td>4.83 x 10⁵*</td>
</tr>
<tr>
<td>D,L-2,3-DCP</td>
<td>29.58*</td>
<td>3.80 x 10⁻⁵ M (0.38 mM)*</td>
<td>7.78 x 10⁵*</td>
</tr>
<tr>
<td>MCA</td>
<td>04.25</td>
<td>2.50 x 10⁻⁴ M (0.25 mM)</td>
<td>1.70 x 10⁴</td>
</tr>
<tr>
<td>MBA</td>
<td>362.50</td>
<td>6.70 x 10⁻³ M (0.67 mM)</td>
<td>5.41 x 10⁵</td>
</tr>
</tbody>
</table>

(*): values corrected for D isomer
3.6.5 Database analysis of dehalogenases by amino acid sequence comparison

The Rhizobial DehL and DehD were completely different from each other by amino acid sequence comparison (18% identity) (Cairns, 1994). The deduced amino acid sequence of DehE also showed little identity to DehD (14%) and DehL (16%) (Stringfellow et al., 1997). It is of interest to compare each of the dehalogenase amino acid sequences from Rhizobium sp. by Blast search (NCBI) programme to see if there is any other protein with a similar amino acid sequence now present in the databases.

The DehL amino acid sequence when compared to the database did not show any sequence similar to it. However, sequence from DehD showed slight identity to D,L-DEX enzyme (18% identity) derived from Pseudomonas sp. strain 113 (Nardi-Dei et al., 1997) (Figure 3.12).

Using the same search, the DehE amino acid sequence was also compared to the database and showed high identity to sequences from Alcaligenes xylosoxidans ssp. DhlIV (72% identity) as shown in Figure 3.13 and Dehl from Pseudomonas putida PP3 (72% identity) as shown in Figure 3.14. DL-DEX from Pseudomonas sp. strain 113 showed 39% identity (Figure 3.15).

Since all these three enzymes were reported to be sensitive to sulphydryl blocking reagents, their protein sequences were compared in order to identify any consensus cysteine residue in the protein (Figure 3.16). The amino acid sequence of DhlIV and Dehl contain two cysteine residues (at position 42 and 288), D,L-DEX contains one cysteine residue (at position 178) and DehE contains 4 cysteine residues (at position 42, 128, 256 and 288). The results showed a conserved cysteine residue between DehE, DhlIV and Dehl at position 42 and 288. However, these cysteines were not seen in DL-DEX enzyme.

Pseudomonas putida PP3 was also reported to have another dehalogenase, DehII (Topping, 1992). This enzyme classified as type 2I (Slater et al., 1997). Although this enzyme is in the same group as DehE there was no sequence identity to DehE. In addition, DehII from Pseudomonas putida PP3 was also compared to DehL or DehD from Rhizobium sp. did not show any matching sequence (data not shown).
Figure 3.12. Amino acid sequence showing regions of identity between DehD from *Rhizobium* sp. and D,L-DEX from *Pseudomonas* sp. strain 113 (Nardi-Dei et al., 1997) (Percent identity 18% and similarity 27%)

Two dots indicate amino acid similarity

Figure 3.13. Amino acid sequence comparison between DehE from *Rhizobium* sp. and DhllV from *Alcaligenes xylosoxidans* ssp. (Brokamp et al., 1997) (Percent identity 72% and similarity 78%)

Two dots indicate amino acid similarity
Figure 3.14. Amino acid sequence comparison between DehE from *Rhizobium* sp. and Dehl from *Pseudomonas putida* PP3 (Topping, 1992).

(Percent identity 72% and similarity 74%)

Two dots indicate amino acid similarity

Figure 3.15. Amino acid sequence comparison between DehE from *Rhizobium* sp. and D,L-DEX from *Pseudomonas sp.* strain 113 (Nardi-Dei et al., 1997).

(Percent identity 39% and similarity 50%)

Two dots indicate amino acid similarity
Figure 3.16. Multiple sequence alignment (Corpet, 1988) of *Rhizobium sp.*


* indicates sequence identity
3.6.6 G+C content

The G+C content for the complete nucleotide sequence for dehL, dehD and dehE was 55%, 56% and 60%, respectively. However, there was no reported G+C value for the complete genome of Rhizobium sp. For that reason, the G+C content of the 16S rRNA of this organism was calculated from both Figures 4.3 and 4.4 and found to be 55% (Chapter 4). This will give an idea of the average of the genomic G+C content of Rhizobium sp. The G+C content for dehE appeared to be somewhat higher than the average of the G+C of the 16S rRNA, whereas G+C for dehL and dehD showed the same value. The results showed the inconsistency of the G+C content between all three dehalogenases might suggest that dehE has been gained independently, possibly via a plasmid.

3.7 Discussion

Leigh (1986) reported that DehD and DehL were stereospecific for D- and L-2CP, respectively, and DehE could act on both enantiomers. The present investigation confirms this stereospecificity as well as the inability of DehD and DehL to react with 2,2DCP. DCA and MCA were confirmed not to be substrates for DehD and DehL, respectively, and neither enzyme could act on TCA.

The three dehalogenases were able to act on D,L-2,3DCP. Using DehE indicated that only chloride from one position was released, presumably from carbon 2 because according to Allison (1981) dehalogenase from Rhizobium sp. did not react with 3CP. Based on this information presumably chloride at carbon 3 was not attacked. The possible product of dehalogenation was proposed to be 2-hydroxy-3-chloropropionate. The total chloride released using DehL or DehD enzyme also indicated that D,L-2,3DCP had equimolar L- or D- isomers similar to D,L2-CP.

The Km values for various substrates using crude cell-free extract and purified dehalogenases did not show any major difference between them. However, purification of all three dehalogenases was carried out to determine the Kcat values. The Kms for growth and non-growth halogenated substrates were also determined (Table 3.5) with pure enzymes. With DehD, D-2CP and D,L2-CP had the lowest Km values of 0.06mM and 0.04mM, respectively. With DehL, L-2CP and D,L2-CP gave 0.15mM and 0.11mM, respectively. However, DehE gave Km values 4x higher for the same
substrate. This suggests DehD and DehL are better enzymes for D,L2-CP. The low Km values also indicate the possibility that the *Rhizobium sp.* might grow at low concentration of halogenated compounds. It will be of particular interest to investigate growth at lower substrate concentration to determine whether the same dehalogenases are involved or new enzyme(s) produced.

DehE enzyme was used to determine the Km values for non-growth substrates (Table 3.4). The results show the Kms for chlorinated and brominated compounds were roughly equivalent. Generally, substitution of other halogens for chlorine increases the Km values due to increase of electronegativity, but this is not the case for DehE. However, the Kms decreased when the number of chlorine or bromine substituents increased.

There is very little information in the current literature regarding dehalogenase Km values. Some of the reported values were very high compared to the current investigation. *Pseudomonas sp.* strain 113 dehalogenase (D,L-DEX) has similar features to those of DehE of *Rhizobium sp.*, in that both act on D- and L-2CP and are classified in Class 2I (Nardi-Dei et al., 1997). However, the amino acid sequence comparison between these two enzymes showed only 39% identity. Pure protein obtained from the expression of the DL-DEX gene in *E.coli* had a molecular weight of 68,000Da and was composed of two identical subunits similar to that of DehE of *Rhizobium sp.* (62,000Da). The calculated Km values of D,L-DEX for L-2CP, D-2CP and MCA were 0.9, 4.2 and 4.8mM, respectively. These values were generally high compared to the Kms of the pure DehE enzyme from *Rhizobium sp.* with 0.56, 0.85 and 1.88mM using the same substrates. However, there are no Km values reported for other dehalogenases from the same group as DehE, DehII (Topping, 1992) and DhIIV from *Alcaligenes xylosoxidans* ssp. *denitrificans* ABIV (Brokamp et al., 1997). The amino acid sequences of DehE and DhIIV showed 72% sequence identity whereas Dehl from *Pseudomonas putida* PP3 also showed high percent identity to DehE (72%) but no Km data has been reported for that enzyme.
The amino acid sequences from all three dehalogenases that act on D,L2-haloalkanoic acid were compared to DehE from *Rhizobium sp.* (Figure 3.16) and showed high sequence identity. In contrast, the DehII enzyme from the same class as DehE did not show any sequence identity to DehE. However, the amino acid sequence for Dehl, which is not in the same class as DehE showed 72% amino acid sequence identity. Based on this fact classification of dehalogenases by substrate specificities according to Slater *et al.*, (1997) can be misleading.

DhIIIV and DehE act on haloalkanoic acids by inversion of the optical configuration (Brokamp *et al.*, 1997; Allison (1981); Leigh *et al.*, 1988) (Class 2I), whereas Dehl maintains the optical configuration of substrate-product (Class 2R). Weightman, *et al.*, (1982) suggested that this mechanism involves an active –SH group from a cysteine residue leading to retention of configuration and enzymes from this group are inhibited by thiol reagents. DehE contains four cysteine residues, DhIIIV and Dehl contain two cysteine residues each and D,L-DEX contains one cysteine residue. When all four sequences were compared, the conserved cysteine residue was detected at position 42 and 288 (Figure 3.16), while D,L-DEX do not coincide with any cysteines present in the other three dehalogenases. According to Janssen *et al.*, (1994) some dehalogenases are still inhibited by thiol reagents, although no cysteine is involved in the dehalogenating reaction. For D,L-DEX it has been confirmed that an aspartic or glutamic acid residue may play an important role in catalysis and the presence of N-ethylmaleimide, *p*-chloromercuribenzoate and iodoacetamide did not cause inactivation of the enzyme suggesting cysteine did not involve in the enzyme catalysis (Nardi-Dei *et al.*, 1997). However, for DehE and DhIIIV (with conserved cysteine residues at position 42 and 288), it is still yet unclear if the cysteines are involved in the mechanism of catalysis. In addition, by looking at the reaction mechanisms between DehE, DhIIIV or Dehl and the classification of these dehalogenases established by Slater *et al.*, (1997), it is questionable whether these three dehalogenases are homologous.

Another organism, *Pseudomonas* AJ1/23, was reported to have two dehalogenase enzymes, which act specifically on the D-isomer and L-isomer of 2-CP. Had-D, which was specific for D-isomer was purified and its kinetic properties studied. The Km value for MCA and D,L2-BP was 27.5 and 1.99mM, respectively (Smith *et al.*, 1990). These values are apparently much higher than those from *Rhizobium sp.* with the
corresponding values for DehD enzyme for MCA of 1.02mM and for D,L-2BP at 0.2mM. The comparison of deduced amino acid sequences between HadD and DehD showed 23% identity as reported by Cairns (1994).

**Kcat values and the Specificity Constants** were calculated for each dehalogenase enzyme using different substrates as shown in Table 3.7(i), (ii) and (iii). The Specificity Constant values have an upper limit of $10^8$ to $10^9$ M$^{-1}$sec$^{-1}$ (Nelson and Cox, 2000). Some enzymes were reported to have values near to the upper limit for example Fumarase, $1.6 \times 10^8$ M$^{-1}$sec$^{-1}$ (Fersht, 1999). However, DehL, DehE and DehD like many enzymes of metabolism have slightly lower values in the range of $10^4$ to $10^5$ M$^{-1}$sec$^{-1}$. The Specificity Constants for D,L2-CP of DehL, DehE and DehD were $2.08 \times 10^5$ M$^{-1}$sec$^{-1}$, $2.86 \times 10^4$ M$^{-1}$sec$^{-1}$ and $1.46 \times 10^5$ M$^{-1}$sec$^{-1}$, respectively suggesting that both DehL and DehD were better catalysts than DehE enzyme with this substrate.

**Rhizobium sp.** was originally isolated using 2,2DCP and it was curious that DehL and DehD were also present since these enzymes were confirmed not to act on 2,2DCP (Leigh, 1986). One possible reason might be the commercially available 2,2DCP was not pure and contained D,L2-CP (Leigh, 1986). A second explanation is possibly that DehL and DehD were chromosomally located and the gene encoding DehE was obtained separately via plasmid transfer.

There are two pieces of evidence to suggest DehE may originally have been plasmid encoded. First, by sequence homology, where the DehE from *Rhizobium sp.* has high identity to the DhlIV from *Alcaligenes xylosoxidans* ssp., which is plasmid located (Brokamp et al., 1997). Second, the inconsistency of G+C content of the nucleotide sequences of *dehE* (60%) compared to the *dehL* (56%) and *dehD* (55%) whose G+C values matched to that of the 16S rRNA (55%) that may represent the total genomic DNA of *Rhizobium sp.*. However, the actual G+C content of the genomic DNA from *Rhizobium sp.* under study has not been determined. As there are differences in the G+C content of *dehE* and the chromosome of its host, *dehE* may not originally have been chromosomally encoded.
The use of dehalogenases is important for industrial biocatalysis, for example in the manufacture of chiral intermediates. D-2-haloacid dehalogenase has found commercial application in the production of L-2CP as a chiral feedstock chemical for the production of herbicides (ICI patent no. 179603) and anti-inflammatory agents (Swanson, 1999). The D-2-haloacid dehalogenase was studied in detail for use in industry for developing a bioreactor system where D-2-haloacid dehalogenase from *Pseudomonas putida* AJI/23 was immobilised. This resulted in increased temperature stability and ability to withstand mildly alkaline conditions (Parker and Colby, 1995). A dehalogenase of opposite stereospecificity, a thermostable L-2-haloacid dehalogenase (L-DEX) enzyme from *Pseudomonas* (Lui et al., 1994) and L-2-haloacid dehalogenase from *Azotobacter* strain RC26 (Diez et al., 1996) have been characterised in terms of their better thermostability and resistance to enzyme inhibitors. The apparent Km for dehalogenase from *Azotobacter* strain RC26 using MCA as a substrate was reported to be 0.18mM close to the Km value of the same substrate using DehD enzyme (0.25mM) from *Rhizobium* sp.. However, there is no reported Km value for L-DEX from *Pseudomonas* (Lui et al., 1994).

Since D-2-haloacid dehalogenase is important in industry compared to the L-2-haloacid dehalogenase, the attractive Km/Vmax values and Kcat values for DehD from *Rhizobium* sp. might suggests that this enzyme may have merit in an industrial process.
CHAPTER 4
INVESTIGATION OF GROWTH OF
RHIZOBIUM SP. AT LOW
CONCENTRATIONS OF HALOGENATED
COMPOUND
4.1 Introduction

In relation to environmental pollution, many investigations have been conducted about the global distribution of persistent organochlorines such as PCBs and DDT. These contaminants are transportable and contaminate all over the world (Iwata, et al., 1993). The destruction of organic chemicals by microorganisms may be influenced by environmental factors or the structure of the chemical itself. One of the reasons suggested for the lack of degradation of organic compounds by microbes is their low concentration (Boethling and Alexander, 1979). Microorganisms that metabolise and grow upon very low concentrations of substrates have been designated as oligotrophs (Poindexter, 1981). Such organisms appear to be adapted to low substrate concentrations by having high substrate affinity (low Km value) systems.

Biodegradation of very low concentrations of xenobiotic compounds has been neglected. However, it is useful to know about growth of microorganisms in low concentrations of pollutants because of the legal requirements. Normally this is set by a government to get the concentration of a pollutant down to a level that is not considered harmful. Therefore, if the microorganisms could only remove high concentration of pollutants, they could not be used to meet requirements of the law, since there still will be low concentrations of pollutants in the environment.

The Rhizobium sp. had been isolated using 20mM 2,2DCP and it was not known whether the organism could grow at concentrations of the order of one hundredth of this value i.e. could exhibit oligotrophism? However, should growth be possible at very low substrate concentrations some of the Km values reported in the previous chapter suggest that the known dehalogenases might function satisfactorily at low substrate concentrations. Alternatively, a different type of dehalogenase with a higher substrate affinity might be needed.

The following section describes confirmation of the genus name of the organism used in this study and investigation of the possibility of growth at low concentrations of halogenated substrate.
4.2 Further confirmation of the bacterium as a *Rhizobium* sp. by 16S rRNA gene sequencing

According to Berry *et al.* (1979) this organism was first identified by standard microbiological techniques as a fast growing *Rhizobium*. In an attempt to confirm the species identity it was subjected to 16S rRNA gene sequence comparison. The first stage was amplification of the gene using PCR. The primers used, which were based on a conserved region of the 16S rRNA gene, are shown in Figure 4.1.

The partial sequence obtained is shown in Figure 4.2. The first 500bp of sequence from both primers was compared with sequences on the EMBL Database using the FASTA 3 tool (Pearson and Lipman, 1988). Based on sequence comparison, for two thirds of the gene, the closest match of 96% identity for both 5' and 3' ends was with *Rhizobium loti* (Sullivan *et al.*, 1996) (Figure 4.3 and 4.4). Examples of other organisms that matched the sequence are shown in Table 4.1. The current sequence information could be used to design internal primers to allow further clarification of the sequence. However, the experiment confirmed the genus of the species is *Rhizobium*, as claimed before.

<table>
<thead>
<tr>
<th>Organisms that closely matched the sequence from both 5' and 3' ends</th>
<th>Percent identity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>R. loti</em> <em>(LMG 6125)</em></td>
<td>94%</td>
</tr>
<tr>
<td><em>Mesorhizobium loti</em></td>
<td>94%</td>
</tr>
<tr>
<td><em>Mesorhizobium</em> sp. USDA 40</td>
<td>94%</td>
</tr>
<tr>
<td><em>Mesorhizobium</em> sp. 'SH 15003'</td>
<td>94%</td>
</tr>
<tr>
<td><em>Mesorhizobium loti</em> DNA</td>
<td>94%</td>
</tr>
<tr>
<td><em>Rhizobium ciceri</em></td>
<td>93%</td>
</tr>
<tr>
<td><em>Rhizobium</em> sp.</td>
<td>93%</td>
</tr>
<tr>
<td><em>Mesorhizobium amorphae</em></td>
<td>93%</td>
</tr>
</tbody>
</table>
Figure 4.1. 16S rRNA gene representation

Forward 16S rRNA primer
FD1: AGA GTT TGA TCC TGG CTC AG
(Position: 8-27bp)

Reverse 16S rRNA primer
rP1: ACG GTC ATA CCT TGT TAC GAC TT
(Position: 1492-1512bp)
Figure 4.2. *Rhizobium sp.* 16S rRNA partial sequence
Figure 4.3. Sequence comparison for 5' region of *Rhizobium sp.* 16S rRNA gene (Percent identity: 96%)

*Rhizobium loti* is one example of the matching sequence from the database search.
**Figure 4.4.** Sequence comparison for 3' region of *Rhizobium sp.* 16S rRNA gene (Percent identity: 96%)

*Rhizobium loti* is one example of the matching sequence from the database search.
4.3 Growth of *Rhizobium sp.* on 0.2mM 2,2-dichloropropionate (2,2DCP)

*Rhizobium sp.* was reported to grow on 2,2DCP, D,L2-CP and D,L2-BP as sole sources of carbon and energy (Berry *et al.*, 1979). However, growth did not occur on 2,2DCP and D,L2-CP at concentrations in excess of 50mM or 20mM respectively, suggesting toxicity of these compounds to the organism (Leigh, 1986). The normal 2,2DCP concentration used to grow *Rhizobium sp.* was 20mM. To investigate the ability of *Rhizobium sp.* to grow on low concentrations of substrate, 0.2mM 2,2DCP, 100x lower than the normal growth concentration, was used. 2,2DCP was chosen because it was widely used as a herbicide (Dalapon).

An initial experiment was carried out to establish whether *Rhizobium sp.* was able to grow on 0.2mM 2,2DCP. The cell inoculum was prepared by growing *Rhizobium sp.* in 20mM lactate minimal medium. Then 15ml of an overnight culture was centrifuged and the cells washed twice with minimal medium before inoculation into 100ml minimal medium supplied with 0.2mM 2,2DCP. Growth was monitored by measuring the amount of chloride ions released at appropriate time intervals. An uninoculated flask treated in the same way was used as a control. This is important to make sure the chloride measured in the growth medium was due to the cells using the 2,2DCP rather than the auto-degradation of the substrate in the growth medium. A typical growth curve is shown in Figure 4.5(a) with a doubling time of approximately 12 hours. From the growth curve the time to harvest cells was set between mid and late logarithmic phase so that at harvesting the cells were still active and producing dehalogenase. For larger scale preparation cells were grown in 8 flasks each with 500ml of medium containing 0.2mM 2,2DCP as a carbon source.

As a control *Rhizobium sp.* grown in 20mM 2,2DCP was also prepared for use in further analysis, as shown in Figure 4.5(b). A doubling time of approximately 11 hours was observed which was more or less the same doubling time for growth at low concentration. Time for harvesting cells was set between mid and late logarithmic phase similar to that for growth at low concentration.
Figure 4.5(a). Chloride released during growth of *Rhizobium sp.* on 0.2mM 2,2DCP

*Rhizobium sp.* : inoculated sample with *Rhizobium sp.*

Control : uninoculated sample

Figure 4.5(b). Growth of *Rhizobium sp.* on 20mM 2,2DCP
4.3.1 Analysis of cell-free extract from 0.2mM 2,2DCP-grown bacteria by enzyme assay

Table 4.2 shows the dehalogenase specific activity for crude extracts prepared from cells grown on 0.2mM 2,2DCP and on 20mM 2,2DCP. The substrates chosen were based on the specificity of the three dehalogenases the organism is known to produce. DBA is a substrate for DehL and DehE; 2,2DCP a substrate for DehE; MCA a substrate for DehD and DehE; and D,L2-CP a substrate for all three dehalogenases. The results suggest that similar dehalogenase enzymes (DehL, DehE and DehD) are produced under the two growth conditions. The dehalogenase specific activity using DBA as substrate for cells grown in 20mM 2,2DCP is twice that seen for growth at 0.2mM 2,2DCP. Since from the 2,2DCP measurement the amount of DehE in each growth is the same, there must be more DehL in the cells grown on 20mM 2,2DCP.

Such assays of crude extract may not show the presence of any additional dehalogenase(s) in cells grown on low concentration, so the crude extracts were checked by Native-PAGE analysis.

Table 4.2. Dehalogenase specific activity for crude extracts from cells grown at low and high concentration

<table>
<thead>
<tr>
<th>Substrates</th>
<th>0.2mM 2,2DCP-grown bacteria (μmolCl/min/mg protein)</th>
<th>20mM 2,2DCP-grown bacteria (μmolCl/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dibromoacetate (DBA)</td>
<td>0.66</td>
<td>1.40</td>
</tr>
<tr>
<td>2,2-dichloropropionate (2,2DCP)</td>
<td>0.065</td>
<td>0.055</td>
</tr>
<tr>
<td>Monochloroacetate (MCA)</td>
<td>0.130</td>
<td>0.110</td>
</tr>
<tr>
<td>D,L-2-chloropropionate (D,L2-CP)</td>
<td>0.22</td>
<td>0.23</td>
</tr>
</tbody>
</table>
4.3.2 Separation of DehL, DehE and DehD on Native-PAGE

The gel electrophoresis technique provides an efficient way of separating mixtures of proteins with different electrophoretic mobility to enable extracts to be analysed. Silver nitrate can be used to locate halide produced by dehalogenase activity in the gel. The separation depends on the size of the protein and the charge of the protein. However, proteins with similar charge and size will not be separated by this procedure.

In preliminary experiments DehD, DehL and DehE were run on a Native gel made using Tris.SCl buffer pH8.8. The gel was then incubated with 50mM D,L2-CP followed by staining the gel with 0.5%(w/v) silver nitrate solution. D,L2-CP was used because all three dehalogenases could react with this substrate. The results showed that DehD and DehL migrated to the same position and DehE moved faster (Figure 4.6). The native molecular weights were close to each other (DehL: 60,000Da; DehE: 62,000Da; DehD: 58,000Da) so that could not account for the different electrophoretic mobility. However, from their amino acid composition DehE shows an overall negative charge whereas DehL and DehD apparently have positive charges (Table 4.3). This may be the reason why DehE moved faster than the other two enzymes. Although the theoretical charge for DehL and DehD was positive they must still have negative charges on the protein surface because both proteins move into the gel but the extent of negative charge could not be calculated from the amino acid composition. It is impossible to know the actual charge for DehL and DehD but presumably it is less than for DehE because DehL and DehD moved more slowly.

Attempts were made to separate DehL and DehD by running the proteins in Native gels prepared from Tris.SO₄ buffer pH7.8 and pH6.8. However, both DehL and DehD still moved in the same relative positions seen in Figure 4.6 and so could not be separated.
Figure 4.6. Native-PAGE analysis of dehalogenase activity
Lane 1: DehL
Lane 2: DehD
Lane 3: DehE

Table 4.3. Estimated charge for dehalogenase enzymes from amino acid composition

<table>
<thead>
<tr>
<th>AMINO ACID</th>
<th>DehL RESIDUES</th>
<th>DehE RESIDUES</th>
<th>DehD RESIDUES</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARG</td>
<td>26</td>
<td>21</td>
<td>30</td>
</tr>
<tr>
<td>LYS</td>
<td>8</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>ASP</td>
<td>14</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>GLU</td>
<td>17</td>
<td>25</td>
<td>11</td>
</tr>
<tr>
<td>Total negative</td>
<td>31</td>
<td>37</td>
<td>23</td>
</tr>
<tr>
<td>Total positive</td>
<td>34</td>
<td>29</td>
<td>33</td>
</tr>
<tr>
<td>Overall</td>
<td>+3</td>
<td>-8</td>
<td>+10</td>
</tr>
</tbody>
</table>
4.3.3 Native-PAGE analysis of the cell-free extract from *Rhizobium sp.* grown on 0.2mM 2,2DCP and on 20mM 2,2DCP

Cell-free extracts of *Rhizobium sp.* grown on 0.2mM and 20mM 2,2DCP were analysed by Native-PAGE. After electrophoresis, the gels were incubated with 2,2DCP, DBA and MCA and then stained for halide ions.

The result shows that when incubated with 2,2DCP a single band seen for the 0.2mM 2,2DCP-grown cells was in the same position as the DehE of the 20mM 2,2DCP grown cell-extract (Figure 4.7a). This suggests DehE was produced when cells were grown on 20mM and 0.2mM 2,2DCP. However, a single band from the cells grown at low concentration might also indicate the presence of a different dehalogenase that migrated to the same position as DehE. However, other dehalogenases might not be detected on the gel if the high concentration of substrate used (50mM) was inhibitory. To check this a Native gel was incubated with 0.2mM 2,2DCP. However, no bands were seen for either of the extracts.

The presence of other known dehalogenases could be detected using other substrates such as DBA and MCA. DBA was a specific substrate for DehE and DehL (Figure 4.7b) and MCA was specific for DehE and DehD (Figure 4.7c). The gel incubated with DBA showed two distinct bands, with different intensities between faster and slower bands for the two growth conditions (Figure 4.7b). Two possible explanations of this might be the relative amount of DehL is less than the amount of DehE when cells are grown at low concentration or a new dehalogenase might be present. The results for cells grown on high concentration might suggest that the relative amount of DehL is more than DehE (Table 4.2). There was no difference in band pattern between the slower and the faster bands for a gel incubated with MCA, as shown in Figure 4.7(c). The band patterns from these figures could not be compared directly with the specific activities calculated in Table 4.2 because different buffer conditions were used in the Native gel and in the enzyme assay.
Figure 4.7(a). Dehalogenase activity towards 2,2DCP
Lane 1: Cell-free extract from 20mM 2,2DCP-grown bacteria
Lane 2: Cell-free extract from 0.2mM 2,2DCP-grown bacteria

Figure 4.7(b). Dehalogenase activity towards DBA
Lane 1: Cell-free extract from 20mM 2,2DCP-grown bacteria
Lane 2: Cell-free extract from 0.2mM 2,2DCP-grown bacteria
Figure 4.7(c). Dehalogenase activity towards MCA

Lane 1: Cell-free extract from 20mM 2,2DCP-grown bacteria
Lane 2: Cell-free extract from 0.2mM 2,2DCP-grown bacteria

(The actual bands were weak and difficult to see on the gel photograph)
As a control experiment gels incubated with distilled water remained clear when stained for chloride or bromide ion, suggesting that the bands seen were due to enzyme substrate interaction. To further characterise the dehalogenase enzyme(s) present, apparent Km values were measured for crude extracts prepared from growth at 0.2mM and 20mM 2,2DCP.

### 4.3.4 Apparent Km values using crude extract prepared from cells grown on 20mM and 0.2mM 2,2DCP

The apparent Km values for extracts prepared from growth at high and low concentration are shown in Figure 4.8(a) and (b) using DBA as substrate. The Km value was 0.18mM in both cases. The Km values are closer to the known Km for DBA of crude DehL (0.19mM) rather than the value for crude DehE (0.89mM).

However, the reason why the apparent Km appears to indicate the presence of DehL rather than DehE could be investigated by looking at the relative amount of each individual enzyme produced from the two growth conditions. This could be achieved by separating them on an anion exchange column assuming no loss of activity occurred.

### 4.3.5 Measurement of the dehalogenase enzyme profile for cells grown on 0.2mM and 20mM 2,2-DCP

Conditions for the separation of a mixture of DehL, DehE and DehD by anion exchange chromatography were established first before attempting to separate extracts from cells grown on low and high 2,2DCP concentration. Cell-free extracts of DehL, DehE and DehD were prepared in low ionic strength buffer, 0.01M Tris-acetate pH7.6, and were mixed together before running on a MonoQ 5/5 column over a 5 to 100mM sodium phosphate gradient. The fractions were collected and dehalogenase activity was measured in each fraction using D,L2-CP as substrate. The elution profile is shown in Figure 4.9. Three peaks of dehalogenase activity were present in this profile, as expected. The enzyme elution depended on phosphate buffer concentration: for DehL (47mM), DehE (72mM) and DehD (31mM). Under this condition, all three dehalogenase enzymes were successfully separated based on their charges. Since DehE protein has more negative charges as shown in Table 4.3 it will be retained more firmly on the column and was eluted last.
Figure 4.8(a). Apparent Km using crude extract from *Rhizobium sp.* grown on high (20mM) concentration of 2,2DCP with DBA as substrate (The crude extract was prepared in 0.1M Tris-acetate pH7.6 buffer)

Figure 4.8(b). Apparent Km using crude extract from *Rhizobium sp.* grown on low (0.2mM) concentration of 2,2DCP with DBA as substrate (The crude extract was prepared in 0.1M Tris-acetate pH7.6 buffer)
Figure 4.9. MonoQ elution profile of a mixture of DehL, DehE and DehD

Extract was loaded onto a MonoQ 5/5 column and eluted with a linear 5 to 100mM sodium phosphate gradient. DehL eluted at 47mM phosphate buffer, DehE eluted at 72mM phosphate buffer and DehD eluted at 31mM phosphate buffer. The elution profile of each dehalogenase in U/mg is superimposed on the protein trace.
DehD and DehL also carried negative charges, otherwise they would not separate using this technique. However, DehD was eluted earlier than DehL and DehE presumably due to the fact that it has less negative charges compared to the other two enzymes. The negative charge on the surface of DehL was presumably less than for DehE. For that reason DehL was eluted before DehE from the MonoQ column. For analysis of extracts obtained from Rhizobium sp. grown at 0.2mM and 20mM 2,2DCP, a similar technique was utilised.

An extract from Rhizobium sp. grown on 0.2mM 2,2DCP was prepared in 0.01M Tris-acetate buffer pH7.6 and applied to a MonoQ column. Protein fractions eluted were screened using D,L2-CP as substrate to detect the presence of DehL, DehE and DehD. The analysis showed that Fraction 9 (DehD), Fraction 13 (DehL) and Fraction 16 (DehE) showed activity. These fractions were then analysed further using DBA as substrate for Fraction 13 (DehL) and Fraction 16 (DehE) and MBA for Fraction 9 (DehD), to determine the activity for each dehalogenase enzyme present. The elution profiles were plotted as in Figure 4.10. The activity of each enzyme was calculated and is shown in Table 4.4 with DehL (0.2U/mg) twice as much as DehE (0.12U/mg) enzyme.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Unit of enzyme/mg (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DehL</td>
<td>0.20</td>
</tr>
<tr>
<td>DehE</td>
<td>0.12</td>
</tr>
<tr>
<td>DehD</td>
<td>0.29</td>
</tr>
</tbody>
</table>

Km values with DBA as substrate were measured for Fraction 13 (DehL) and Fraction 16 (DehE). The results showed that the Km for Fraction 13 (0.10mM) was similar to the Km for crude DehL (0.19mM) and the Km for Fraction 16 (0.47mM) was similar to the Km for crude DehE (0.89mM) (Figure 4.11a and b) supporting the identification of the activities.
Figure 4.10. MonoQ elution profile of a crude extract prepared from *Rhizobium sp.* grown on 0.2mM 2,2DCP

Extract (1ml: approximately 3.5mg) was loaded onto MonoQ 5/5 column and eluted with a linear 5 to 100mM sodium phosphate gradient. DehD eluted at 33mM, DehL eluted at 54mM and DehE eluted at 73mM. The elution profile of each dehalogenase in U/mg is superimposed on the protein trace.
Figure 4.11(a). Km value of Fraction 13 (growth on 0.2mM 2,2DCP) using DBA as substrate

Figure 4.11(b). Km value of Fraction 16 (growth on 0.2mM 2,2DCP) using DBA as substrate
This result suggested that growth on low concentration of 2,2DCP did not produce any new dehalogenase. Table 4.4 shows the activity of DehL produced was more than DehE and this possibly explained the reason for the apparent Km with the crude extract being close to that of DehL (Figure 4.8 b). However, this finding still did not indicate why the intensity of the faster band was stronger than the slower band from cells prepared from growth at low 2,2DCP concentration as shown in Figure 4.7(b).

To further investigate this matter, the crude extract from growth on 0.2mM 2,2DCP that was run on the MonoQ column was subjected to Native-PAGE and stained for dehalogenase activity with DBA. The results showed that the faster and the slower bands stained to the same intensity in this case (Figure 4.12). This was in contrast to the result seen in Figure 4.7(b) where the intensity of the faster band was stronger than the slower band. The only difference between the two conditions was the concentration of buffer used to make the crude extracts. For Figure 4.7(b), 0.1M Tris-acetate buffer pH7.6 was used and for Figure 4.12, 0.01M Tris-acetate buffer pH7.6 was used and the extract was ultra-centrifuged.

To see whether the difference was due to the different concentration of buffer used to make the extract another batch of cells from the same growth experiment on 0.2mM 2,2DCP was resuspended in 0.1M Tris-acetate buffer pH7.6, sonicated and ultracentrifuged similar to the sample preparation for the MonoQ column. When this extract was run on a Native-PAGE gel it showed stronger intensity in the faster band compared to the slower band (Figure 4.13) similar to the earlier results shown in Figure 4.7(b). This suggested that the band pattern seen on the gel depended on the concentration of buffer used to make the extract. However, the crude extract was not prepared in 0.1M Tris-acetate pH7.6 buffer for the MonoQ column because all three dehalogenases would not then bind to the column.

Using the extract that was prepared in 0.01M Tris-acetate pH7.6 buffer, the apparent Km value was determined using DBA as a substrate to see whether extract prepared in this different buffer condition affected the Km. The Km value 0.21mM (Figure 4.14a), was close to the Km for the same substrate using DehL enzyme (0.19mM).
Figure 4.12. Native-PAGE analysis of crude extract from *Rhizobium sp.* grown on 0.2mM 2,2DCP extracted in 0.01M Tris-acetate buffer pH7.6.

Figure 4.13. Native-PAGE analysis of crude extract from *Rhizobium sp.* grown on 0.2mM 2,2DCP extracted in 0.1M Tris-acetate buffer pH7.6.
Figure 4.14(a). Apparent Km value using DBA as substrate for crude extract from *Rhizobium sp.* grown on 0.2mM 2,2DCP extracted in 0.01M Tris-acetate buffer pH7.6

![Graph showing Km value for crude extract from Rhizobium sp. grown on 0.2mM 2,2DCP extracted in 0.01M Tris-acetate buffer pH7.6.]

Km $0.21 \pm 0.09$

Figure 4.14(b). Apparent Km value using DBA as substrate for crude extract from *Rhizobium sp.* grown on 0.2mM 2,2DCP extracted in 0.1M Tris-acetate buffer pH7.6

![Graph showing Km value for crude extract from Rhizobium sp. grown on 0.2mM 2,2DCP extracted in 0.1M Tris-acetate buffer pH7.6.]

Km $0.18 \pm 0.05$
However, the $K_m$ (0.21mM) is only slightly more than the $K_m$ for DBA using cell-free extract prepared in 0.1M Tris-acetate buffer pH7.6 (0.18mM) (Figure 4.14b). This analysis suggests that preparing the cell-free extract in 0.1M Tris-acetate buffer pH7.6 or in 0.01M Tris-acetate buffer pH7.6 did not effect the apparent $K_m$, significantly.

As a comparison an extract from *Rhizobium sp.* grown on 20mM 2,2DCP was analysed in an identical way. The specific activity of the cell-free extract prepared in 0.01M Tris-acetate pH7.6 buffer was checked using D,L2-CP as substrate (Specific activity: $0.20\mu\text{molCl}^{-1}/\text{min/mg protein}$) to make sure that the cell-free extract contain dehalogenase activity before loading on a MonoQ column. The fractions were screened using D,L2-CP to detect the most active fractions. The active fractions were eluted at 39mM (Fraction 9), 54mM (Fraction 13) and 73mM (Fraction 16) sodium phosphate buffer corresponding to DehD, DehL and DehE, respectively (Figure 4.15). They were then further analysed by enzyme assay using DBA and MBA as substrates to determine the activity of each enzyme present. The results showed that the relative activity of DehL produced was 3.5x more than DehE (Table 4.5). The $K_m$ value determined using DBA as substrate for Fraction 13 (0.20mM) was close to the $K_m$ using crude DehL (0.19mM) and $K_m$ for Fraction 16 (0.57mM) was close to the $K_m$ using crude DehE (0.89mM) (Figure 4.16ab).

The dehalogenase profiles from growth on 0.2mM and 20mM 2,2DCP suggested that different relative activity of each dehalogenase enzyme were produced in the two growth conditions (Table 4.5). The data indicates that more activity in DehL than DehE for both growth conditions and as a result the apparent $K_m$ measured in the cell-free extract is likely to represent primarily the activity of DehL. The different relative activity of each dehalogenase enzyme produced were possibly due to different gene expression at high and low substrate concentration.
Figure 4.15. MonoQ elution profile of a crude extract prepared from *Rhizobium sp.* grown at 20mM 2,2DCP

Extract (1ml: approximately 4mg protein) was loaded onto a MonoQ 5/5 column and eluted with a linear 5 to 100mM sodium phosphate gradient. DehD eluted at 32mM, DehL eluted at 54mM and DehE eluted at 73mM. The elution profile of each dehalogenase in U/mg is superimposed on the protein trace.
Figure 4.16(a). Km value of Fraction 13 (growth on 20mM 2,2DCP) using DBA as substrate

Figure 4.16(b). Km value of Fraction 16 (growth on 20mM 2,2DCP) using DBA as substrate
Table 4.5. Dehalogenase activities in cells grown on 0.2mM 2,2DCP and 20mM 2,2DCP concentration

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Unit of enzyme/mg (Growth on 0.2mM 2,2DCP)</th>
<th>Unit of enzyme/mg (Growth on 20mM 2,2DCP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DehL</td>
<td>0.20</td>
<td>0.78</td>
</tr>
<tr>
<td>DehE</td>
<td>0.12</td>
<td>0.22</td>
</tr>
<tr>
<td>DehD</td>
<td>0.29</td>
<td>0.56</td>
</tr>
</tbody>
</table>

4.4 Discussion

The original identification of *Rhizobium* sp. based on biochemical tests and morphological observations (Berry et al., 1976) was extended by the analysis of the 16S rRNA. The determination of 16S rRNA gene sequences is a routine procedure in prokaryotic taxonomy, resulting in large and growing databases, which improve phylogeny reconstructions, identification results and primer specificity evaluations (Nubel et al., 1997). The 16S rRNA gene sequence analysis confirmed that the organisms showing the highest identity are various *Rhizobium* species. However, the analysis was not extensive enough to identify the species name of the organism.

According to Lane et al., (1985) in assessing the relationship of one organism to another by the comparison of their 16S rRNA sequence it is not important that the complete sequences be determined. The search in the database showed the sequence from the 5’ and 3’ matched the same organisms as in Table 4.1. The sequences from these organisms matched to the *Rhizobium* sp. ranging from 96% to 94%. This suggests the organism used in this study is from the genus *Rhizobium*.

There is only one reported case associated with hydrolytic dehalogenation which involves the genus *Rhizobium*. The halidohydrolase from this organism was able to attack chlorosubstituted s-triazine (Bouquard et al., 1997). Rhizobia is a collective name of the genera *Rhizobium, Sinorhizobium, Mesorhizobium* and *Bradyrhizobium*. *Agrobacterium* sp. from *Rhizobium* subdivision was reported capable of complete
mineralisation of 2,3-dichloro-1-propanol (Effendi et al., 2000). Generally, it is quite rare for organisms from \textit{Rhizobium sp.} to be involved in hydrolytic dehalogenation. However, this kind of organism is always associated with bacteria of agronomic importance because they form nitrogen-fixing symbioses with leguminous plants (Kaneko et al., 2000) and the majority of genes for nitrogen fixing symbioses seem to be present on the chromosome of \textit{Mesorhizobium} and \textit{Bradyrhizobium} (Kundig, et al., 1993; Sullivan and Ronson, 1998; Kaneko et al., 2000).

The \textit{Rhizobium sp.} was able to grow on 0.2mM 2,2DCP with a doubling time of approximately 12 hours, similar to the growth rate obtained at 20mM 2,2DCP concentration indicating that the organism was a facultative oligotroph.

Dehalogenases produced during growth on 0.2mM 2,2DCP were analysed by electrophoreotic mobility on Native-PAGE and protein separation by ion exchange chromatography to investigate whether growth at low concentration may involve a new dehalogenase enzyme. The electrophoreotic patterns of dehalogenase activity staining using DBA showed that cells grown on low concentration and on high concentration produced the same electrophoreotic band pattern suggesting that the same dehalogenases were produced but the relative intensity of the bands was apparently different. However, it is difficult at this stage to explain the reason why the intensity of the faster band was different from the slower band in extracts prepared from growth at lower 2,2DCP concentration compared to growth at higher 2,2DCP concentration. Maybe further investigation needs to be carried out to determine the N-terminal sequence of each dehalogenase that was present from growth at low 2,2DCP concentration to check its identity.

The Native gel electrophoresis also shows that there was more than one dehalogenase enzyme produced during growth on high and low 2,2DCP concentration. This observation was similar to the previous investigation by Leigh (1986) when \textit{Rhizobium sp.} grown on 20mM 2,2DCP as sole source of carbon and energy revealed three dehalogenases although DehL and DehD did not play any role in 2,2DCP degradation. In other cases variation in growth condition influenced the nature of dehalogenase production (Hardman and Slater (1981a). According to Allison \textit{et al.}, (1983) different inducers gave different pattern of induction compare to 2,2DCP and it was seen in the
current investigation that different activity of dehalogenases were observed when growth was at high and low 2,2DCP concentration.

Analysis by column chromatography strongly suggests that DehL activity was more than DehE in each growth condition. This might explain why the apparent Km values using the crude extract were close to the Km of DehL for DBA. In addition, to support this finding, previous evidence from *Pseudomonas putida* PP3 has shown that the nature of the growth substrate does affect the relative proportions of two dehalogenase made by that organism as judged by the observed activity ratios (Slater *et al.*, 1979). Different growth conditions might also suggest a complex regulatory control in *Rhizobium sp.*, which responded to the growth environment.

The present investigation did not show any new dehalogenase from growth at low haloalkanoic acid concentration. The low Km value for 2,2DCP of DehE (0.19mM) as reported in the previous chapter would seem to make that enzyme suited to dealing with low concentrations of growth substrate and no other enzyme would appear to be necessary.
CHAPTER 5
CLONING OF *RHIZOBIUM SP.* PUTATIVE DEHALOGENASE REGULATOR GENE
5.1 Introduction

Rhizobium sp. growing on halogenated alkanoic acids such as 2,2DCP was found to produce three haloalkanoate dehalogenases (Leigh et al., 1988). Mutant analysis suggested that formation of the enzymes was controlled by a single regulator gene (Leigh, 1986). It was curious that the organism had more than one dehalogenase when one of them (DehE) on its own could act on all the identified substrates of the other two dehalogenases. In fact, of the three dehalogenases, only DehE could utilise 2,2DCP on which the organism was isolated.

Regulation of dehalogenase gene expression in Rhizobium sp. is poorly understood. Leigh (1986), proposed a possible model for the control of the three dehalogenases. It was suggested that gene expression was positively regulated since a presumed single point mutant strain was unable to produce any of the three dehalogenases. To study further the regulator gene it is appropriate to isolate the gene from the Rhizobium sp. chromosomal DNA and clone it into a vector in order to characterise the gene product.

5.2 Detecting the Rhizobium sp. putative dehalogenase regulator gene by a probing method

5.2.1 Choosing a gene probe

Stringfellow et al., (1997) successfully cloned and sequenced the dehE gene (Figure 5.1). Upstream of the dehE gene on the insert DNA of pSC520 was a sequence that was tentatively identified as part of a dehalogenase regulator gene by deduced amino acid sequence comparison with a dehalogenase regulator protein from Pseudomonas putida PP3 (Stringfellow et al., 1997).

The restriction enzyme sites present in the putative regulator gene sequence identified using the DNA Strider programme, indicated that a 210bp EcoRI and XhoI internal fragment was present (Figure 5.2). The presence of this fragment was confirmed by restriction enzyme digestion and it was isolated from an agarose gel for use as probe for the dehR gene.
Figure 5.1. Nucleotide sequence and derived amino acid sequence of \textit{dehE} gene and a truncated \textit{dehR} gene (Stringfellow, et al., 1997). Sequence in the box indicates the 210bp probe region.
Figure 5.2. Restriction site map of pSC520 insert DNA

Key to enzymes: E: EcoRI; B: BamHI; Sa: SalI; S: Sall; H: HindIII; Bg: BglII; X: XhoI.

Arrows indicate the direction of transcription of the dehE gene and the putative dehR gene.

Black line: 210bp EcoRI/XhoI probe
5.2.2 Dot blot assay to detect \textit{dehR}

In this test the labelled probe was found to hybridise with the chromosomal DNA from the \textit{Rhizobium sp.} (Figure 5.3a). The negative control (calf thymus DNA) showed no hybridisation while the positive control (pSC520) showed a dark spot (Figure 5.3b). From the dot blot assay, the \textit{Rhizobium sp.} chromosomal DNA contained the target sequence, as expected.

5.2.3 Probing chromosomal DNA to identify the \textit{dehR} gene

A restriction enzyme fragment needs to be identified that will contain the whole \textit{dehR} gene (Figure 5.4). Firstly, it is necessary to estimate how large the \textit{Rhizobium sp.} regulator gene is likely to be. If the \textit{Rhizobium sp.} regulator gene is a similar size to the \textit{Pseudomonas putida} PP3 regulator gene it would be about 1.7 kb. So, 2kb was used as a rough estimate for the size of fragment required. From the known locations of particular restriction enzyme sites in the region, it is possible to deduce what size of a particular restriction fragment would be needed to include the whole of the regulator gene.

The pSC530 restriction map (see Figure 5.4) shows the presence of \textit{PstI}, \textit{HindIII}, \textit{SalI}, \textit{BamHI}, and \textit{SacI} sites in the 5' region of the putative \textit{dehR} gene. For fragments to include the 2kb \textit{dehR} gene using these enzymes the size would have to be 2kb for the gene plus the distance from the particular site to the start of the \textit{dehR} gene. This size is for: \textit{SalI} 2.5 kb, \textit{SacI} 3kb, \textit{BamHI} 3kb, \textit{HindIII} and \textit{PstI} 4kb. Thus, if \textit{HindIII} is used to digest completely the \textit{Rhizobium sp.} chromosomal DNA, the probe would need to hybridise to a fragment of 4kb or above. The same idea will apply for other restriction enzyme digests. For a \textit{BamHI} fragment to include the regulator gene the probe needs to hybridise at 3kb or above, for \textit{SalI} at 2.5kb or above, for \textit{SacI} at 3kb or above and for \textit{PstI} at 4kb or above.

In the first investigation chromosomal DNA was totally digested with \textit{SalI}, \textit{SacI} and \textit{PstI}, respectively, and run out on a 0.8% agarose gel, then probed with the labelled probe. The \textit{SalI} digested sample gave a band that hybridised at about 4kb, the \textit{SacI} digested DNA hybridised at 2kb while the \textit{PstI} digest gave a fragment that hybridised at 3kb (Figure 5.5ab). As mentioned before, for \textit{PstI} digests to include the whole \textit{dehR} gene the hybridising fragment should be 4kb or more for this restriction
Figure 5.3. (a) Dot blot assay of Rhizobium sp. chromosomal DNA

No.1  Rhizobium sp. chromosomal DNA (2.4µg)
No.2  Rhizobium sp. chromosomal DNA (1.5µg)

Figure 5.3. (b) Dot blot assay

No.1  Negative control – calf thymus DNA (2.0µg)
No.2  Positive control – pSC520 (0.2µg)
Figure 5.4. Restriction enzyme sites identified that may give a fragment that contains the whole dehR gene

The diagram is based on the restriction map of pSC530 that contained dehE and putative dehR gene fragment.

Key to enzymes: P: PstI; H: HindIII; S: Sall; B: BamHI; Sa: SacI

Arrow: direction of transcription of the putative dehR gene

Black line: 210bp EcoRI/XhoI probe
(P, H, S, B, Sa): indicates possible restriction enzyme sites 3' to the dehR region
Figure 5.5. (a) Agarose gel showing a total digest *Rhizobium sp.* chromosomal DNA
Lane 1: 1kb DNA ladder; Lane 2: *PstI* digest; Lane 3: *SacI* digest and Lane 4: *SalI* digest. The DNA was then transferred onto a nitrocellulose membrane before hybridisation with the labelled probe.

Figure 5.5. (b) X-Ray film showing hybridised chromosomal DNA
Lane 1: 1kb DNA ladder
Lane 2: Chromosomal DNA digested with *PstI* hybridised at 3kb
Lane 3: Chromosomal DNA digested with *SacI* hybridised at 2kb
Lane 4: Chromosomal DNA digested with *SalI* hybridised at 4kb
site not to be present within the regulator gene itself. The size of 3kb suggested that there is an internal PstI site in the dehR gene. For SacI digests to include the whole regulator gene a band needs to hybridise at 3kb or more. However, the result showed that the probe hybridised at 2kb indicating that an internal SacI site was present within the regulator gene.

A SalI fragment hybridised at 4kb, which therefore seems likely to include the desired complete dehalogenase regulator gene. So the 4kb fragments from Rhizobium sp. chromosomal DNA digested with SalI were used to construct a gene library with pUC18 as vector.

5.2.4 Construction of a genomic DNA library

To isolate the dehalogenase regulator gene, a genomic library was constructed in E.coli JM109 and the transformants screened by colony hybridisation using a 210bp EcoRI and XhoI probe. For this, Rhizobium sp. genomic DNA was totally digested with SalI restriction enzyme and size selected fragments in the 4-5 kb range were used as DNA insert because this size is expected to contain the dehalogenase regulator gene. Before ligation, the extracted DNA was probed to make sure the hybridising fragment was present.

5.2.5 Screening for dehalogenase regulator gene by ‘colony’ hybridisation

For colony hybridisation the transformant colonies were grown on LB/ampicillin/IPTG/X-gal plates. The library was screened using the non-radioactive labelled probe. To differentiate between positive results and negative results in the screening process positive and negative controls were included on the same plate. The positive control was pSC520 (the source of the probe) in JM109. The positive control shows dark patches. The negative control (pUC18) without insert DNA in JM109 shows no hybridisation (Figure 5.6).

Figure 5.6 shows an example of the cell patches screened using the EcoRI-XhoI probe. Some of the patches were dark (numbers 133, 142, 84). These patches were therefore likely to contain the desired insert and needed to be further investigated.
Figure 5.6. Colony hybridisation of gene library sample

Cells marked as No. 133, 142 and 84 appeared positive

Positive control: pSC520

Negative control: pUC18
However, when the plasmid DNA was prepared and the restriction enzyme digests carried out none of the plasmids contained the desired fragment. Approximately 2000 colonies were checked in this way but none of them carried the required insert.

In this investigation colony hybridisation was found to be problematic. Since this approach was unsuccessful, an alternative method of identifying the gene was used. This involved construction of a gene library and phenotypic selection for the presence of the dehE gene in the expectation that the adjacent dehR gene would be co-isolated. This will be described in the following section.

5.3 Cloning the putative regulator gene together with the dehE gene

This method of selection will detect a new ability of the host cell to grow on a substrate that previously it was unable to grow on. Two main criteria need to be met to achieve this. First, the host needs to be able to take up into the cell the carbon source used for the selection and second the host needs to be able to use the product of dehalogenation as a source of carbon and energy. Dehalogenation of D,L-2CP by DehE gives rise to lactate, a normal growth substrate for E.coli.

A specific uptake system for halogenated compounds is not expected to be present in the E.coli host. But the uptake of D,L2-CP could possibly occur via a lactate uptake system that might be induced by D,L2-CP in the growth medium. This is due to the chemical structure and the size of lactate being similar to that of chloropropionate. For that reason D,L-2CP was used as a carbon source to identify the desired clone.

5.3.1 Construction of a genomic library

As shown previously an approximately 4kb fragment is needed to include both dehE and dehR genes. Chromosomal DNA was digested either with HindIII or PstI. These restriction enzymes were chosen because according to the pSC530 restriction map such a fragment may include both the dehE gene and the putative dehR gene (Figure 5.7). Chromosomal DNA digested with HindIII gave a fragment of 4kb that hybridised with the probe, as shown in Figure 5.8 (a,b) which was slightly bigger than that seen for the PstI digest. Therefore, only the 4kb HindIII fragment region was extracted from an agarose gel and ligated into pUC18 to produce a gene library.
Figure 5.7. Predicted restriction fragment to include the complete *dehE* and *dehR* genes

*Xhol* and *EcoRI* fragment (solid line) is the 210bp probe. The *dehR* gene is estimated to be about 1.7kb to 2.0kb in length. (H?, P?) is unknown location of *HindIII* and *PstI* sites in the 3' region of *dehR*. The size of the fragment required to include both genes is 4kb or above.

Arrows: direction of transcription

Key to enzymes:
P: *PstI* ; H: *HindIII* ; S: *SalI* ; X: *Xhol* ; E: *EcoRI*
Figure 5.8. (a) Agarose gel showing a total digest of *Rhizobium* sp. chromosomal DNA prior to blotting for Southern analysis
(Approximately 5μg of DNA were digested completely before being run on a 0.8% agarose gel)
Lane 1: 1kb DNA ladder
Lane 2: Chromosomal DNA digested with *Hind*III
Lane 3: Chromosomal DNA digested with *PstI*

Figure 5.8. (b) X-Ray Film showing hybridised chromosomal DNA
Lane 2: *Hind*III digest / hybridised at 4kb fragment
Lane 3: *PstI* digest / hybridised at 3kb fragment
5.3.2 Growth of transformants on D,L2-CP

The gene library was transformed into *E.coli* XL10 Gold supercompetent cells (Stratagene). To improve transformed cell viability, they were first grown in 1.5ml LB for one hour at 37°C. Then ampicillin (to a final concentration of 100μg/ml) was added. Following incubation at 37°C overnight the cells were harvested, washed with sterile distilled water, resuspended in sterile distilled water and plated out onto selective D,L2-CP minimal medium plates and incubated at 30°C. IPTG was included to act as an inducer in case the *dehE* gene is under control of the *lac* promoter. Purified D,L2-CP was used in the plates because *E.coli* K-12 might be inhibited by impurities in non-treated D,L2-CP (Cairns, 1997).

After 4 days incubation individual colonies that grew were transferred onto a fresh D,L2-CP plate. A single colony (1a) from this plate was then grown on LB/amp for plasmid DNA preparation.

5.3.3 Restriction mapping and Southern analysis

The plasmid prepared from this isolate (1a) was analysed by restriction enzyme digest. Through a series of single and double digests it was possible to confirm the previous map of pSC520 and the location of the newly cloned putative regulator gene. However, in order to reconfirm the restriction digest of the plasmid, Southern Blot analysis was also carried out. For this analysis it was possible to predict the fragment size that would include the putative regulator gene that would hybridise to the probe. The restriction pattern obtained can be seen in Figure 5.9(a). Figure 5.9(b) indicates the fragments that hybridised with the *EcoRl* - *XhoI* probe.

From Figure 5.9(b) it can be seen that a single hybridisation band is present at 3.8kb in the *HindIII* digests at Lane 4. There was no hybridisation to the 2.7kb vector DNA. The plasmid digested with *EcoRI* (Lane 5) showed the 2.5kb fragment hybridised with the probe as expected but not the 3.9kb fragment. In the double digest (Lane 6) with *EcoRI* and *HindIII*, the same size (2.5kb) fragment hybridised but not the 2.7kb of the vector or the 1.5kb fragment. In Lane 7 the *SalI* digest showed the 4.5kb fragment hybridised. Because there are three *SalI* sites the larger fragment that hybridised included the vector. The two fragments of approximately 1.4kb and 200bp did not
Figure 5.9. (a) Fragment analysis of plasmid 1a prior to blotting for Southern analysis

Positive control (Lane 1) pSC520 digested with HindIII; 1kb DNA ladder (Lane 2, 3 & 8); plasmid 1a digested with: HindIII (Lane 4); EcoRI (Lane 5); EcoRI/HindIII (Lane 6); Sall (Lane 7)

Figure 5.9. (b) X-Ray film of hybridised fragments

Lane 1  pSC 520 hybridised at 4.8kb (positive control)
Lane 2, 3 & 8  1kb DNA ladder (negative control);
Lane 4  Plasmid 1a/HindIII hybridised at 3.8kb but not 2.7kb
Lane 5  Plasmid 1a/EcoRI hybridised at 2.5kb but not 3.9kb
Lane 6  Plasmid 1a/EcoRI/HindIII hybridised at 2.5kb but not at 2.7kb and 1.3kb
Lane 7  Plasmid 1a/Sall hybridised at 4.5kb but not 1.4kb and 200bp
hybridise. Figure 5.10 shows the deduced restriction map based on the restriction enzyme digests that were then confirmed by Southern analysis (Figure 5.9b). The plasmid was designated pFH648.

5.3.4 Subcloning of pFH648

From the data obtained from restriction enzyme mapping it could be seen that there were useful sites from which subclones could be constructed. The purpose of the subcloning was to separate the \textit{dehE} gene from the regulator gene.

The first subclone constructed involved the deletion of approximately 2.2kb to leave a 2.0kb \textit{SalI} – \textit{HindIII} fragment, from map position 2.0 to position 4.0kb, that carried the putative \textit{dehR} sequence (Figure 5.11). For this pFH648 was digested with \textit{SalI} and the desired fragment was extracted from the agarose gel. The purified DNA fragment was then re-ligated and transformed into competent \textit{E.coli}. Transformed colonies were selected on the basis of antibiotic resistance. Restriction analysis of the miniprepped plasmid showed that it contained the expected insert and so the plasmid was designated pFH45. Experiments to determine the \textit{dehR} gene sequence by automated DNA sequencing were then carried out.

5.3.5 Nucleotide sequencing of the putative regulator gene

A major aim of this project was to obtain a complete sequence of the \textit{Rhizobium sp.} dehalogenase regulator gene, then to express the gene and study the regulatory protein. The nucleotide sequencing was carried out on the plasmid pFH45.

The initial sequence was obtained by using standard M13 forward and reverse primers. The full sequence on both strands was obtained using custom-synthesised oligodeoxyribonucleotide primers. These were designed from the sequence already obtained (Figure 5.12 and Table 5.1).

By obtaining the whole of the regulator gene sequence the derived amino acid sequence could then be compared with the derived amino acid sequence of \textit{Pseudomonas putida} PP3 dehalogenase regulator protein.
Figure 5.10. Restriction map of pFH648 to locate the putative *dehR* gene

Polylinker region (E-H) not to scale (left hand side). Arrows denote direction of transcription for both *dehE* and *dehR* genes.

Key to enzymes:
H: HindIII; S: SalI; X: XhoI; E: EcoRI
Figure 5.11. Subclones to show *dehR* gene in pFH45

Thick line: *dehR* gene

Hatched area: indicates polylinker region of pUC18 (not to scale)

Key to enzymes:

H: *HindIII*; X: *XhoI*; S: *SalI*; E: *EcoRI*
Figure 5.12. The use of custom designed oligodeoxyribonucleotide primers to sequence the entire dehalogenase regulator gene

Thick line: dehR gene

Hatched region indicates: multicloning region.

Key to enzymes: S: SalI; X: XhoI; E: EcoRI; Sa: Sacl; H: HindIII
Table 5.1. Custom-designed oligodeoxyribonucleotide primers for sequencing the dehalogenase regulator gene

<table>
<thead>
<tr>
<th>Name of oligodeoxyribonucleotide</th>
<th>Sequence of oligodeoxyribonucleotide 5’ to 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13 reverse</td>
<td>CAG GAA ACA GCT ATG ACC</td>
</tr>
<tr>
<td>Reg2R</td>
<td>GCC ATC TTC TAC CGC GAT CGC ATG</td>
</tr>
<tr>
<td>Reg3R</td>
<td>GCA ATT GCA CAA CTT ATA CAC CGC</td>
</tr>
<tr>
<td>Reg4R</td>
<td>GCA ACG TGA GAG AGC TCC GCA ACC</td>
</tr>
<tr>
<td>M13 forward</td>
<td>TGT AAA ACG ACG GCC AGT</td>
</tr>
<tr>
<td>Reg2L</td>
<td>CGA CTC CAG ATC CTT GTT GGT GGC</td>
</tr>
<tr>
<td>Reg3L</td>
<td>CGC GAG CAC GCT CTT TCC AGT ACG</td>
</tr>
<tr>
<td>Reg4L</td>
<td>CGT CAA AAT GGT GTC GCC GTG AAT</td>
</tr>
</tbody>
</table>

5.3.6 Putative dehalogenase regulator gene sequence

The complete sequence of the insert of pFH45 is given in detail in Appendix II including sites of restriction endonucleases. The nucleotide sequence of the dehR gene was then converted into a 556 amino acid sequence for comparison with the *Pseudomonas putida* PP3 regulator protein sequence.

5.3.7 The sequence analysis of *Rhizobium sp.* dehalogenase regulator gene

Stringfellow *et al.*, (1997) identified the first ORF of the 1.3kb *SalI* fragment from pSC520 as the *dehE* gene (Figure 5.2). The second, truncated, ORF was identified as a putative dehalogenase regulator gene starting 217bp from the *SalI* site (Figure 5.1 and 5.2) upstream of the *dehE*. The first 83 amino acids of the second ORF were compared to the databases and there was a significant identity (51%) and similarity (74%) when conservative substitutions were taken into account, to the N-terminal region of a *Pseudomonas putida* PP3 dehalogenase regulatory gene protein (DehR<sub>i</sub>) (Topping *et al.*, 1995).
To find out the extent of the identity between the putative *Rhizobium sp.* dehalogenase regulator protein and that from *Pseudomonas putida*, the whole amino acid sequence of the dehR gene was then compared. The relatedness of the proteins can be seen from the alignments in Figure 5.13. The sequence comparison gave 70% identity. However, there was no stop codon in the putative *Rhizobium* dehalogenase regulator gene sequence. Therefore, it suggests that the full *Rhizobium* regulator gene may not have been cloned. It is possible that the *Rhizobium sp.* dehR is longer or shorter than that of *Pseudomonas putida* PP3. Attempts to clone any additional sequence will be described next.

5.3.8 Cloning the missing dehR gene sequence

As mentioned before the lack of a stop codon in the sequence suggests the putative dehR gene was not complete. Having a complete dehR sequence was critical for production of DehR protein for subsequent investigations. A probe for the missing sequence was the 230bp *SacI-HindIII* terminal region (Figure 5.14).

To clone the possible missing fragment, *Rhizobium* chromosomal DNA was digested with *SacI* plus a series of other restriction endonucleases and probed with the 240bp *SacI-HindIII* fragment to identify, hopefully, a small (approximately 500bp) fragment that would include the sequence after the HindIII site (Figure 5.14).

Figure 5.15 (a) and (b) show that by Southern blot analysis restriction fragments that might include the sequence were identified. The fragment (1kb) nearest to 500bp was seen in the *SacI-PstI* digest in Lane 2. The *SacI-KpnI* digest shows a fragment of about 2kb which was larger than required, *SacI-SmaI* digests showed two hybridising fragments. This might be due to incomplete digestion of the chromosomal DNA with those enzymes. The *SacI-Sau3AI* fragment appeared to be the same size as the *SacI-HindIII* fragment and so would probably not carry the required DNA. The *SacI-PstI* 1kb fragment was then cloned to determine the missing dehR gene sequence.
Figure 5.13. Amino acid sequence comparison between *Rhizobium* sp. putative dehalogenase regulator protein and *Pseudomonas putida* PP3 regulator protein sequence (70% identity).

Two dots denote amino acid similarity.
Figure 5.14. Possible restriction fragments that will include the sequence downstream of the *HindIII* site

Restriction enzymes used are: *KpnI*, *Sau3Al*, *SmaI* and *PstI*. These enzymes had no sites in the *SacI/HindIII* region of pFH45.

The *SacI/HindIII* fragment was used as a DNA probe.

Key to enzymes:
- Sa – *SacI*
- H – *HindIII*
- K – *KpnI*
- P – *PstI*
- S – *SmaI*
Figure 5.15. (a) Agarose gel showing a total digest of *Rhizobium* sp. chromosomal DNA prior to blotting for Southern analysis.

Chromosomal DNA digested with: *SacI/KpnI* (Lane 1); *SacI/PstI* (Lane 2); *SacI/Smal* (Lane 3); *SacI/Sau3Al* (Lane 4); 1kb DNA ladder (Lane 5).

Figure 5.15. (b) X-ray film of hybridised membrane.

Chromosomal DNA digested with: *SacI/KpnI* hybridised at 2kb (Lane 1); with: *SacI/PstI* hybridised at 1kb (Lane 2); with: *SacI/Smal* hybridised at 1kb and 2.3kb (Lane 3); with: *SacI/Sau3Al* hybridised at 200bp (Lane 4); 1kb DNA ladder (Lane 5).
5.3.9  Cloning of 1kb SacI / PstI fragment
Chromosomal DNA was completely digested with SacI/PstI and the 1kb region was extracted from the gel. Before ligation into pUC18 vector, the 1kb region was probed (SacI-HindIII probe) to confirm that the desired fragment was present. However, colony hybridisation to detect the desired clone in the gene library was again found to be problematic as described before. Colonies that hybridised with the probe, when tested further showed no hybridisation of the restricted plasmid DNA.

5.3.10  PCR of ligation mix
A possible method for identifying the desired SacI/PstI fragment was to use PCR methodology. In the ligation mixture, the SacI / PstI fragment should have been ligated into the pUC18 vector as shown in Figure 5.16. To amplify this region the Reg2 specific primer was designed from the known dehR sequence between SacI and HindIII (5'gcg gca aaa aat ttg gga gta age 3'). The other primer for the 3’end was the standard forward M13 primer of pUC18. Results from the PCR show as expected, the desired 1kb fragment using the specific Reg2 primer and the standard forward M13 primer. Controls leaving one of the primers did not show any band pattern. Unexpectedly, using the specific Reg2 primer and the standard reverse M13 primer an approximately 1kb band was also obtained, but it was slightly bigger.

5.3.11  Sequencing of the PCR product
The 1kb product amplified using Reg2 primer and standard M13 forward primer was sent for automated sequencing. The sequencing primer used was Reg2. The sequence obtained shows 9 bases of the sequence within the SacI/HindIII region, which then extends beyond the HindIII site. This suggests that the PCR product is the desired continuation from the HindIII restriction site (Figure 5.17). A stop codon was located after 36 bases from the HindIII site.

5.3.12  Sequencing of the ligated HindIII fragment
Another possible way of sequencing the fragment was to digest the PCR product with HindIII and ligate it into pUC18. Plasmid DNA was prepared from such a potential clone and checked using SphI to see the possible orientation of the cloned DNA. Based on the digest the possible restriction enzyme site map is shown in Figure 5.18. The plasmid carrying the desired DNA was designated pFH100.
Figure 5.16. A possible ligation of the $SacI/PstI$ fragment in pUC18

PCR methodology used to detect the presence of the insert DNA. Solid line indicates the sequence of interest.

Key to enzymes:
E: EcoRI; Sa: $SacI$; P: $PstI$; H: HindIII; Sp: SphI

Hatched region indicates pUC18 polylinker

Figure 5.17. Partial sequence from the PCR product

The nine bases before HindIII are the same as those seen in the $SacI/HindIII$ sequence from pFH45. The sequence in lower case after HindIII represents the missing 3’ end of the full length $dehR$ gene with a ‘tga’ termination codon
There are two orientations which are likely to occur, (i) and (ii):

(i) was designated as pFH100 and was sent for sequencing using a standard reverse primer.

(ii) the same DNA insert but in the opposite orientation

The thick line: the sequence of interest

Key to enzymes:
E: EcoRI; Sa: SacI; P: PstI; H: HindIII; Sp: SphI

Hatched indicates multicloning region
This plasmid was then sent for sequencing using standard reverse primer only. The sequence after the HindIII site of the PCR product and pFH100 were also compared and found to be identical. The nucleotide sequence was translated and found to contain a stop codon after 36 bases, identical to that of the PCR product described before.

5.3.13 The complete nucleotide sequence of the dehR gene

The complete nucleotide sequence of the dehR gene is shown in Figure 5.19. The initiation codon was ATG (methionine). The ribosome-binding site (RBS) (Shine and Dalgarno, 1975) upstream of the initiation codon is shown in bold and underlined.

The reading frame of the Rhizobium sp. dehR gene consisted of 1704 bp, which encoded a 567 amino acid protein with a calculated subunit molecular weight of 64kDa (Table 5.2). Comparison with the Pseudomonas putida PP3 regulator protein (Table 5.2) shows most of the residues are essentially similar in number with less than 2% difference. However, for Isoleucine, Leucine, Asparagine and Glutamine the difference between the two sequences was more than 5%.

5.3.14 Sequence comparison

The amino acid sequence of the Rhizobium sp. putative DehR was also compared to the sequences in the SWISS-PROT - EMBL database. There was high identity with Pseudomonas putida PP3 regulator protein DehR₁ (Topping et al., 1995) (Figure 5.20) that showed 72% sequence identity and 77% similarity when conservative amino acid substitutions were taken into account. However, there was a centrally located gap (residues 246-272) of 27 amino acids within the sequence where the sequence did not match at all. The dehR gene from Rhizobium sp. was re-sequenced using the PCR product that was used to clone it into the pT7-7 plasmid vector (Chapter 6). The sequence was exactly the same suggesting that the Rhizobium sp. sequence was correct.
Figure 5.19. The complete dehalogenase regulator gene sequence

Shine Dalgarno ribosomal binding site is printed in bold and underlined. The new sequence printed in lower case letters indicates the 12 amino acids that complete the sequence.
RLRLERKVASVDGFVWE
951 CGGCTAGGGTCGACGCAGCTGGACGCCCGAGGCTGCTGGTACGGTGGA 1000

SDMLEKLVRLLAQRLAK
1001 AAGCGGACGAGATGCTCAAGAGTTTACGGCTCGCAAGCTGGCAAGG 1050

VDTTLILITGESVGKEA
1051 TCGATACGACGTCTGATAACCGGGAGGCTGTTGTTGGCAGAAGGCA 1100

IAQLIHRESDPGRGRRFI
1101 ATTGCACAACTTATACCCGCGGAAGCCGACCAGGCCCAGGGAACG 1150

KINCAIPGELLESEL
1151 CAAATCAACTGCGGTCCCTACCTGGCAAGTCCAGTCGAGTCGAGC 1200

FGYERGAFTGSSRQGKL
1201 TCACATATGCCGCGGTGCGTTACCCGCTGGAGCCGCCAGGAAAGCTG 1250

GLELADKGTLFLDEIG
1251 GGGTTACGAACTCGCCGACAAGGCGACGTGTGTCTGAGACGATCCG 1300

EMPLDLQVKLQLVLQD
1301 GAGATGCCTCTAGATCCTCAGCTAAACCTGCTTCTCAGTCGGTACCG 1350

KSFTRTVGGGTTDIHVDFR
1351 AGTCATTCCTCGCAGGGTACCAGACACCACAGATGTGCTTGAAGCTC 1400

VVSTATNDLESLSRST
1401 GTCGTCACGGACGACAAGATCTGGAGATCGTGTCTGAGAAGCTAC 1450

FREDLYYRLSVVPLKV
1451 TTTAGGGAGAGATCTCTACTACCTCGTCTCGGGTCACGGTGAAAGTCG 1500

PPLRDRQEDVDVPLLDHF
1501 CACCGCTGCACCAGACACGAGAAGCAGTCGTCCTCTGCTGATCATT 1550

LAENFDNYNFTKRFSEK
1551 CTCCGAGGATTTCAACGACGCTATAACTTATCAGCGTTTTCTCGGAA 1600

VMQRLLEHTWPGNRE
1601 GGTGATGAGCTGCTCTTGAGGACACCACTGGCCGGGCAACGTGAGAGC 1650

LRNLVVERLVVTAPAIEV
1651 TCCGGACACCTGGGAGGCTTTAGTGTTGACGGGCTCTGCAGAACTT 1700

DLSLPDKLAPSFAEDI
1701 CAGCTGAGTTCCGGGCGACACGTCTGGCCAGCTCATTTGTCTGAGACT 1750

PENFDQAAAVAAAYERK
1751 TCCGGAGAATTTCCAGTATTTCAGCGGCTTGCGGCTCACGAAGAAAC 1800

LVQAAATMKYGTLREAAK
1801 TCGTCAGGGCCGCGGACCATGAAATACGCGCCTCTCGGCGAAGGCGGCA 1850

HindIII

NLGVSESTIKRKLRAEH
1851 AATTTGGGAGTAAACGAGATCAAGAGTAAAGGCGAAAGCTTGAGCA 1900

DSRADWZ
1901 tgacagttagggcggattgagctga 1925
Table 5.2. Predicted amino acid composition of the *Rhizobium sp. dehR* gene compared to the *Pseudomonas putida* PP3 regulator protein

<table>
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<th>AMINO ACID</th>
<th><em>Rhizobium sp.</em> / RESIDUE PER–SUBUNIT</th>
<th><em>Pseudomonas putida</em> PP3 /RESIDUE PER–SUBUNIT</th>
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<tr>
<td>Alanine (A)</td>
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<td>Cysteine (C)</td>
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<td>4</td>
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<td>1</td>
</tr>
<tr>
<td>Number of amino acids</td>
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<td>571</td>
</tr>
<tr>
<td>Calculated molecular weight</td>
<td>63 935 Da</td>
<td>64 184 Da</td>
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Figure 5.20. Amino acid sequence comparison between a complete Rhizobium sp. putative regulator sequence and Pseudomonas putida PP3 regulator sequence (Topping et al, 1995). The percent identity is 72% and similarity 77%. Two dots indicate amino acid similarity.
Another sequence that also matched the *Rhizobium sp.* DehR was a dehalogenase regulator (DhlR) from *Xanthobacter autotrophicus* GJ10 (van der Ploeg and Janssen, 1995) (Figure 5.21). However, the identity was only 48% in this case. The 27 amino acid region of non-identity shown in Figure 5.20 was not seen in this comparison with *Xanthobacter autotrophicus* GJ10. The regulator protein sequences of *Pseudomonas putida* PP3 and *Xanthobacter autotrophicus* GJ10 were also compared to see the extent of identity between them. The percent identity was 47%, as shown in Figure 5.22. The alignment of all three regulator sequences is shown in Figure 5.23. Most of the amino acids that were identical were in the central region with some at the C-terminal region.

The protein encoded by DehR was also found to have identity to proteins from the family of transcriptional activators (NifA family 43%; NtrC family 45%) which activate expression from -24/-12 promoters together with the alternative sigma factor $\sigma^{54}$ (Morett and Segovia, 1993). The highest identity was in the central region with some at the C-terminal domain (data not included).

### 5.3.15 Sequence comparison between DehR and DehL, DehE and DehD

The regulator protein was believed to interact with inducer (D,L2-CP) which is also the substrate for dehalogenases. Therefore, the amino acid sequence comparison between DehR and dehalogenases was carried out to see any common sequence between them. There were some sequences in common but no large regions of identity (Figures 5.24a,b,c).
Figure 5.21. Amino acid sequence comparison between *Rhizobium* sp. putative regulator sequence and *Xanthobacter autotrophicus* GJ10 DhlR regulator sequence (van der Ploeg and Janssen, 1995). The percent identity is 48% and similarity 58%. Two dots indicate amino acid similarity
**Xanthobacter**

1. .............................................. M 1

**Pseudomonas**

51. LEQENALMVTMTPNMAKVRNIDGAIYYRDQLP...GKVTLLV 96

**Xanthobacter**

2. GTRATRDPQPPPLSER...DFGEVRNSFQGaFVADGEGRTLLVPNGCE 49

**Pseudomonas**

97. PVAYMLRNEFQELQRLQKYEFLERVHNCYGDIYVADQGRGKTLWLNFE 146

**Xanthobacter**

50. RNYDIRAADWGRP VSDLEADG11RP VIAPRVI AS GERVTAIQRTHKGKT 100

**Pseudomonas**

147. RAYGVSRQFDQGARELREGYAKPLTLWVSTGKRITVHKTNTGKS 196

**Xanthobacter**

150. QLREEQGAGGPVVHGETTRIADLL..RRVGSADTVLTLTGSEGVTGKEV 248

**Pseudomonas**

197. VLAQILFDEAGRVVRVIINSRDTELQALQAEELSRGDLARAQTEVA 149

**Xanthobacter**

152. FARFVHRESARSKAPFIKINCGALPRDLIESELFGYEGAFTGAQRGKP 298

**Pseudomonas**

198. IAKLVHINESDRKLEGRLIKINCGAIPEQLLESELFGYEGAFTGOSNQGKP 346

**Xanthobacter**

248. GMIEMANTGTLFLDEIGELPLDMQVKLLHVLQDRIIARALGATRSLPLDIR 279

**Pseudomonas**

249. GLLELADKGTGLDEIGEMPLDQVLQKLQVQDKFTRVGTITVHVDPR 396

**Xanthobacter**

298. VVAATNRLAKAVETGAFRGLDFYRLNVVPVVPPLRERDDDLPILPLRA 347

**Pseudomonas**

397. V1AAATNRDLKEDVSARAFREDLYRSLSVPLKVPPLERQEDVPLLEHF 446

**Xanthobacter**

348. LASFNQYCTAKQLSHAAARTLVADNPQNIRELRNMERLVTVSVDVI 397

**Pseudomonas**

447. LAEFNKRHHSFDFELEGYQQLLEHSWPQNRKLWRLVLRVLVSVPTDII 496

**Xanthobacter**

398. DVQDL...A1PAAPPRGAGGLEEQVFRFESMALIEDARLRCITRRAAR 444

**Pseudomonas**

497. GTNLLEKQLAPGFFSEDPSAGLDQFAAVAAYERKLARAIDYKGSIEA 546

**Xanthobacter**

445. DLRSQSTIVKLKGGFAA...A 465

**Pseudomonas**

547. NLAISESTVVRKLRMDAESDLNADZ 572

---

Figure 5.22. Amino acid sequence comparison between *Pseudomonas putida* PP3 regulator sequence and *Xanthobacter autotrophicus* GJ10 regulator sequence. The percent identity is 47% and similarity 56%.

Two dots indicate amino acid similarity.

---

157
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Figure 5.23. Multiple sequence alignment (Corpet, 1988) of *Rhizobium sp. DehR* with *Pseudomonas putida* PP3 DehR₁ (Topping *et al.*, 1995) and *Xanthobacter autotrophicus* GJ10 DhlR (van der Ploeg and Janssen, 1995). * indicates sequence identity.
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<td>MKGNDGGSVS ALEALPMAVL EIHGDTITMN RAAALFGQR YGSAELARD</td>
<td>............... ............... ............... MSLK KRIKALTFDT GTTVARLAVP</td>
</tr>
<tr>
<td>NSGVRTLTA DTYEDPLEIF SNCYDGIYVA DGTGKLWLNL EGFERAYGLS</td>
<td>............... ASEMILLRQOA AGETESIET... GRYWPMNCRR RSMQAMLNLG REPPPHTTLM</td>
</tr>
<tr>
<td>NPSGVVTLa DTYEDPLEIF SNCYDGIYVA DGTGKLWLNL EGFERAYGLS</td>
<td>............... ASEMILLRQOA AGETESIET... GRYWPMNCRR RSMQAMLNLG REPPPHTTLM</td>
</tr>
<tr>
<td>RDHFIGRdar ELERLGYAKP LITKVISTG KRITVTHKTR TGKVSLITGV</td>
<td>............... LREQFSLDAl LAEGLDLVDi DDDRAHCWDa PASFDPGAVD DGLAARLDRY</td>
</tr>
<tr>
<td>RDHFIGRdar ELERLGYAKP LITKVISTG KRITVTHKTR TGKVSLITGV</td>
<td>............... LREQFSLDAl LAEGLDLVDi DDDRAHCWDa PASFDPGAVD DGLAARLDRY</td>
</tr>
<tr>
<td>PLFNKHGKVR KVIVNSRDM. TELFQLREQL DQAEDKLDARY ESEELRRLS</td>
<td>............... IAVSFTFVSHi LIIIITTSVIT FLMWWMRSL VREWVSTSHC QQICESGGY</td>
</tr>
<tr>
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<td>............... IAVSFTFVSHi LIIIITTSVIT FLMWWMRSL VREWVSTSHC QQICESGGY</td>
</tr>
<tr>
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<td>............... SRKALRNL WSHAIVSiL M...QRETSAS QQPLINRFDE WQKAIPOCF</td>
</tr>
<tr>
<td>RKVASVGDFV WESDEMLAVL RKAQRLAKVD TTTLITGESG VGKEAIAQLi</td>
<td>............... SRKALRNL WSHAIVSiL M...QRETSAS QQPLINRFDE WQKAIPOCF</td>
</tr>
<tr>
<td>HRESDPRGRR FIKINCGAIP GELLESELFG YERGAFSGS RGKQAOGLL</td>
<td></td>
</tr>
<tr>
<td>HRESDPRGRR FIKINCGAIP GELLESELFG YERGAFSGS RGKQAOGLL</td>
<td></td>
</tr>
<tr>
<td>ADKGTLFLDE IGEMPLDLQV KLLQVLQDKS PTVGAPTDTI HVDPRVTAT</td>
<td></td>
</tr>
<tr>
<td>ADKGTLFLDE IGEMPLDLQV KLLQVLQDKS PTVGAPTDTI HVDPRVTAT</td>
<td></td>
</tr>
<tr>
<td>NKDLESIVSS RTFREDLYR LSVVPLKVPP LRDRQEDVVP LLDHFHAETF</td>
<td></td>
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<tr>
<td>NKDLESIVSS RTFREDLYR LSVVPLKVPP LRDRQEDVVP LLDHFHAETF</td>
<td></td>
</tr>
<tr>
<td>DRYNPTKCFs EKVQLRLLEH TWPQVRELH MLVERLVVTa PAVDLSSL</td>
<td></td>
</tr>
<tr>
<td>DRYNPTKCFs EKVQLRLLEH TWPQVRELH MLVERLVVTa PAVDLSSL</td>
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</tr>
<tr>
<td>PDKLAPSEFA DIPENFDFQA AAVAYERKLv QAATMKYGTL REAAKNLQV</td>
<td></td>
</tr>
<tr>
<td>PDKLAPSEFA DIPENFDFQA AAVAYERKLv QAATMKYGTL REAAKNLQV</td>
<td></td>
</tr>
<tr>
<td>ESTIKRLRA EHDSRADWT2</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 5.24(a). Amino acid sequence comparison between DehR and DehL.**

The percent identity is 17%
Figure 5.24(b). Amino acid sequence comparison between DehR and DehE.

The percent identity is 16%
DehR: MKGNDCGSVS ALEALPMAVL EIHGDITITMN RAARALFGGR YGSVAELARD
DehD: ..............................................................

DehR: NPSGVVLTLA DTYEDPLEIF SNCYGDIYVA DGTGTKLWLN EGQFAYGLS
DehD: ..............................................................

DehR: RDHFGQDAR ELERLGYAKP LITWKVISTG KRITVTHKTR TGKSVLATGV
DehD: ..............................................................

DehR: PLFNKHGKVR KVIVNSRDMT ELFQLREQLD QAEKDLAYE SEEELRLRER
DehD: ..............................................................

DehR: RDHFQDRGDAR ELERLGYAKP LITWKVISTG KRITVTHKTR TGKSVLATGV
DehD: ..............................................................

DehR: PLFNKHGKVR KVIVNSRDMT ELFQLREQLD QAEKDLAYE SEEELRLRER
DehD: ..............................................................

DehR: KVASVDGFVW ESDEMLKVLR LAQRLAKVDT TLLITGESGV KEAIAGQLIH
DehD: ..............................................................

DehR: RESDPRGRRF IKINCGAIPG ELLESELFGY ERGDAFTGSSR QOKLGLLELA
DehD: ..............................................................

DehR: DKGTLFLDEI GEMPLDLQVK LLQVLQDKSF TRVGGTDIHI VDFRVTAT
DehD: ..............................................................

DehR: NKDLESLVSS RTFREDLYYR LSWPLKVPP LRDRQEDWP LLDHFLAEFN
DehD: ..............................................................

DehR: DRYNFTKRFS EKVMQRLLEH TWPGNVR..E LRNLVERLVV TAPAEVIDLS
DehD: ..............................................................

DehR: SLPDKLAPSF AEDIPENFDI QAA.....AVA AYERKLVQA A TMKYGTLRSA
DehD: ..............................................................

DehR: AKNLGVSEST IKRKLRAEHD SRADWTZ....
DehD: ..............................................................

DehR: ..............................................................
DehD: ..............................................................

DehR: ..............................................................
DehD: ..............................................................

DehR: ..............................................................
DehD: ..............................................................

DehR: ..............................................................
DehD: ..............................................................

DehR: ..............................................................
DehD: ..............................................................

DehR: ..............................................................
DehD: ..............................................................

Figure 5.24(c). Amino acid sequence comparison between DehR and DehD.
The percent identity is 17%
5.4 Discussion

Mutant analysis had suggested that all three *Rhizobium sp.* dehalogenase genes were under the control of a single regulatory gene (*dehR*). The *dehR* was proposed to encode a regulator protein which positively controlled dehalogenase formation at the transcriptional level. A model was proposed in which the *dehR* product is an activator protein, which in the absence of inducer may not bind to the promoter region of the structural gene, and therefore transcription does not occur (Leigh, 1986).

The protein encoded by *Rhizobium sp. dehR* was compared with the EMBL/SWISS-PROT database. The results showed the highest identity with the *Pseudomonas putida* PP3 dehalogenase regulator protein with 72% identity, supporting the earlier suggestion that upstream of *dehE* was a *Rhizobium sp.* regulator gene. However, a centrally located gap (residues 246-272) of 27 amino acids where the residues did not match at all was identified (Figure 5.20). A possible explanation of this might be that different amino acids are required to bind to the different promoter regions of the two organisms. However, a similar inducer, D,L2-CP was able to induce production of dehalogenase in both organisms. Another possible reason may be that either of the sequences was not correct, but *dehR* from *Rhizobium sp.* was re-sequenced using the PCR product and the sequence was correct. The DhlR regulator protein sequence from *Xanthobacter autotrophicus* GJ10 also matched the DehR from *Rhizobium sp.* with sequence identity of 48% (Figure 5.21), but the 27 amino acid region of non-identity shown in Figure 5.20 was not seen.

The derived amino acid sequence of DehR from *Rhizobium sp.* also matched with other σ^54^ dependent activators, for example NifA (43%) and NtrC (45%), where mainly the central region shows high sequence identity with some also at the C-terminal region. These observations are similar to those seen when the putative regulator sequence from *Rhizobium sp.* was aligned with DehR_1_ from *Pseudomonas putida* PP3 and DhlR from *Xanthobacter autotrophicus* GJ10 (Figure 5.23). It has been proposed by Drummond *et al.*, (1986) that the central region is responsible for interactions with RNA polymerase and ATP binding (Thöny and Hennecke, 1989).
DehR₁ nucleotide sequence and deduced DehR₁ protein sequence from *Pseudomonas putida* PP3 also showed sequence identity with positive regulatory activator proteins (σ<sup>54</sup>-dependent catabolic gene activators) NtrC of *Klebsiella pneumoniae* (Ronson et al., 1987), NifA typeII of *Rhizobium meliloti*, XylR of *Pseudomonas putida* and DmpR which regulates *dmp* operon associated with dimethyl phenol catabolism in *Pseudomonas putida* (Shingler et al., 1993), all showing approximately 50% sequence identity. NtrC and NifA are two regulatory proteins which stimulate the expression of genes regulated for nitrogen assimilation and nitrogen fixation, respectively.

Since DehR from *Rhizobium sp.* shows high identity (72%) to DehR₁ of *Pseudomonas putida* PP3, the DehR regulator protein from *Rhizobium sp.* may be grouped in the same family of transcriptional activators that regulate transcription from the -24/-12 promoter.

*Alcaligenes xylosoxidans* ssp. *denitrificans* ABIV carries a plasmid with an associated dehalogenase regulatory sequence, *dhlR<sub>IV</sub>*, upstream of a D,L-haloalkanoic acid dehalogenase *dhlIV* (Brokamp, et al., 1997). Brokamp, et al., (1997) also reported that the deduced amino acid sequence of the *dhlR<sub>IV</sub>* resulted in about 95% sequence identity with the *Pseudomonas putida* PP3 regulatory protein (Topping et al., 1995). However, the actual sequence of *dhlR<sub>IV</sub>* was not available in the EMBL database to compare with *Rhizobium sp.* dehR gene product.

The sequence comparison between *Rhizobium sp.* DehR and the DehL, DehE and DehD show some sequences in common (Figures 5.24 a,b,c). The sizes of the regulator gene product estimated from DNA sequencing data and dehR gene expression (approximately 64kDa) was about twice the size of the dehalogenase proteins (between 30 to 32kDa). According to the computer generated alignment DehL matched mainly at the amino terminal half of the regulator protein, whereas between DehR and DehE or DehD, matched at the carboxyl terminal half. However, the significance of this observation was difficult to interpret.

At this stage there is no other evidence to suggest that dehR from *Rhizobium sp.* is a regulator gene except by sequence comparison that gave high identity to *Pseudomonas putida* PP3. However, three pieces of evidence suggested that the dehR<sub>1</sub> of *Pseudomonas putida* PP3 was a regulator gene, which encoded an activator for
expression of the dehalogenase structural gene (Thomas et al., 1992b). First, an E. coli strain containing a transposon, which carries dehI without dehR1 did not produce DehI (this also indicated that an E. coli regulatory system was not involved in dehI expression, and also transcription of dehI from the vector promoter was not possible). Second, a complementation experiment showed DehI activity when plasmids containing both dehI and dehR1 genes were present (transcription of dehI from vector promoter was not possible). This suggests the dehR1 region to the right of dehI probably encoded a trans-acting activator protein transcribed from a promoter within the mobile DEH element. Third, the absence of dehI transcription in dehI containing plasmid even with dehR1 present in an rpoN mutant of P. putida KT2440 indicated that activation by DehR1 protein required the RNA polymerase factor σ54 (Kohler et al., 1989). σ54 is required for expression from -24/-12 promoters (in the up-stream region of dehI) and the binding of σ54 to the promoter sequence requires activation by a specific regulatory protein (Collado-Vides et al., 1991). The common features of the dependence on σ54 and a -24/-12 promoter of related regulatory proteins has been shown in connection with genes for a variety of functions including metabolic, catabolic and fermentations pathways, transport systems, pilin formation and pathogenecity (Thöny and Hennecke, 1989).
CHAPTER 6

ANALYSIS OF *RHIZOBIUM SP.*

DEHALOGENASE REGULATOR GENE

PRODUCT IN *E. COLI*
6.1 Introduction

The dehalogenase regulator gene was cloned and sequenced as described in the previous chapter. However, the complete gene was in two separate clones. The full coding sequence is required to obtain the complete protein and efficient expression is needed to produce the protein in appropriate amounts to be used in further studies. This chapter describes construction of the full dehR gene by PCR methodology and in vivo and in vitro investigations to study its role in dehalogenase gene expression.

6.2 Strategy for over-expression of the dehalogenase regulator gene

To obtain over-expression of the dehR gene, the vector pT7-7 (Tabor, 1985), a derivative of the pBR322 family of vectors, was used. This vector carries a T7 Ø10 promoter. The pT7-7 vector has a multiple cloning site, as shown in Figure 6.1, which contains a recognition site for the restriction endonuclease NdeI. Cleavage of pT7-7 with this enzyme and the ligation of the insert DNA with the appropriate cohesive ends results in the formation of an in frame ATG initiation codon. Expression of the inserted gene is then controlled by the T7 Ø10 promoter. Expression of the target gene is dependent on the provision of T7 RNA polymerase. The bacterial host expression strain BL21(DE3) contains a copy of the T7 RNA polymerase gene under lacUV5 control. When the plasmid is transformed into the expression strain and IPTG added T7 RNA polymerase is produced which in turn controls the expression of the gene of interest on the plasmid.

6.2.1 Engineering of the regulator gene into the pT7-7 vector

Restriction mapping and sequence analysis of the coding sequence of the regulator gene revealed that it did not contain any NdeI and SalI sites (Appendix III). This meant the PCR product derived from copying the gene from Rhizobium sp. chromosomal DNA could be digested with NdeI / SalI without the dehR gene being restricted. The primers used for PCR to copy the dehR gene (Materials and Methods section 2.13, Table 2.3) allowed introduction of the required NdeI and SalI sites. The source of the DNA template used was a 4kb SalI digested chromosomal DNA from Rhizobium sp.
Figure 6.1. Restriction site map of vector pT7-7

bla - β lactamase gene
Ø10 - T7 promoter
rbs - ribosome binding site
6.2.2 Cloning of the PCR product

The gel-purified PCR product was digested with *Nde*I and *Sal*I and ligated into pT7-7 vector obtained by digesting pJS771(*dehE*+) with the same enzymes to remove the *dehE* gene.

The ligation mixture was then used to transform BL21(DE3) for plasmid selection. Plasmid DNA was prepared from selected transformants and digested with *Nde*I and *Sal*I to identify the desired construct (Figure 6.2). One of these constructs was further analysed by sequencing to confirm that the DNA had ligated correctly in frame and to see whether any mutations were introduced by PCR. The results showed the coding sequence was identical to that in Figure 5.19. The pT7-7 vector with the regulator gene insert was named pFH772(*dehR*+).

6.3 Expression of the regulator gene from pFH772(*dehR*+) in *E.coli* BL21(DE3)

6.3.1 Growth and induction in LB/Amp

BL21(DE3) [pFH772] (*dehR*+) was initially induced with a final concentration of 0.3mM IPTG at 30°C. Analysis by SDS-PAGE showed the protein was in inclusion bodies. In an attempt to obtain soluble protein the cells were grown to an *A*$_{600}$nm of approximately 0.5 at 30°C and then induced with IPTG (final concentration of 0.01mM) and allowed to grow for another 20 hours at room temperature (21°C). Control cells were grown in the same conditions except that pJS771 in BL21 (DE3) carrying *dehE* was used.

SDS-PAGE analysis of the fractionated crude extracts is shown in Figure 6.3(a). A strong band at 64kDa was observed on the gel from the resuspended pellet. This corresponded closely to the DehR deduced molecular weight 63 935Da as estimated in Table 5.2. Crude extracts from BL21(DE3) pJS771 showed a strong band at 32kDa as expected for the DehE protein (Figure 6.3b) but did not show the 64kDa protein band, suggesting that the 64kDa protein was due to the inserted *dehR* gene in pFH772.

The DehR protein was found exclusively in the pellet fraction of cell lysates where inclusion bodies are expected. The protein in these inclusion bodies is most probably inactive due to misfolding.
Figure 6.2. Plasmid pFH772 digested with Nacl/SalI showing the 1.7kb DNA insert and 2.5kb vector.

Lane 1: 1.7kb insert and 2.5kb vector DNA; Lane 2: 1kb DNA ladder.
Figure 6.3(a). SDS-PAGE analysis of proteins expressed by *E. coli* BL21 (DE3)* [pFH772]*

Lane:
1- SDS VII marker proteins
2- Pellet BL21 (DE3) [pFH772]— grown without IPTG
3- Pellet BL21 (DE3) [pFH772]— induced with 0.01 mM IPTG
4- SDS VII marker proteins
5- Supernatant BL21 (DE3) [pFH772]— grown without IPTG
6- Supernatant BL21 (DE3) [pFH772]— induced with 0.01 mM IPTG

Figure 6.3(b). SDS-PAGE analysis of proteins expressed by *E. coli* BL21 (DE3)* [pJS771]*

Lane:
1- Supernatant BL21 (DE3) [pJS771]
2- SDS VII marker proteins
3- Pellet BL21 (DE3) [pJS771]
In an attempt to minimise formation of inclusion bodies the cells were grown as described before but without adding IPTG (Figure 6.3a). However, the 64kDa protein still appeared to be in the insoluble fraction of the cells. Although the 64kDa protein was produced in the absence of added IPTG the amount of protein produced with IPTG was more than without IPTG, suggesting that \textit{dehR} gene expression was influenced by IPTG. One reason for the expression without IPTG is that the LB may contain an inducer. This can be checked by growing cells with the same plasmid in minimal medium.

In further investigations, growth and expression was also carried out using LB/amp supplemented with 20mM D,L2-CP. It was hoped that the DehR protein would bind with D,L2-CP and avoid formation of inclusion bodies. The results showed that the protein formed was still in inclusion bodies (Figure 6.4).

\textbf{6.3.2 Growth and induction in minimal medium}

To investigate the effect of the growth media on expression of the dehalogenase regulator gene the cells were grown on 20mM lactate minimal medium. Cells were grown up to $A_{680nm} \ 0.5$ at 30°C before adding IPTG (final concentration of 0.01mM), then allowed to grow for another 20 hours at room temperature (21°C).

Supernatant from cell extracts was analysed by SDS-PAGE and showed no protein band at 64kDa. In contrast, the resuspended pellet showed a strong protein band of approximately 64kDa. No such protein was seen in cells grown without added IPTG (Figure 6.5). This experiment showed that the expression of the \textit{dehR} gene in pT7-7 was controlled by IPTG. The difference in expression without IPTG in minimal medium compared to the cells grown on LB suggested that there might be another factor involved in the gene expression in LB medium. This could be due to some component in LB media acting as an inducer of the T7RNA polymerase or maybe due to the faster growth rate on LB media having an effect on the expression of the gene.

To investigate further this induction in an attempt to minimise formation of inclusion bodies the cells were also grown in 20mM lactate minimal medium as a carbon source in the presence of 20mM D,L2-CP and 0.01mM IPTG. D,L2-CP was included in the hope that it would bind with the regulator protein and allow this protein to fold
Figure 6.4. Expression of *dehR* by BL21(DE3)[pFH772] (*dehR*') on LB/amp medium

Approximately 10μg of protein was loaded into each well.

Lane:

1. Supernatant BL21 (DE3) [pFH772]– induced at 21°C with 0.01mM IPTG
2. Supernatant BL21 (DE3) [pFH772]– induced at 21°C with 0.01mM IPTG in the presence of 20mM D,L2-CP
3. Pellet BL21 (DE3) [pFH772]– induced at 21°C with 0.01mM IPTG
4. Pellet BL21 (DE3) [pFH772] – induced at 21°C with 0.01mM IPTG in the presence of 20mM D,L2-CP
5. SDS VII marker proteins
Figure 6.5. SDS-PAGE showing production of DehR by *E. coli* BL21 (DE3) [pFH772] (*dehR*) in 20mM lactate minimal medium

Approximately 4μg of protein was loaded into each well

Lane:

1 - Supernatant BL21 (DE3) [pFH772] – with 0.01mM IPTG
2 - SDS VII marker proteins
3 - Pellet BL21 (DE3) [pFH772] – with 0.01mM IPTG
4 - SDS VII marker proteins
5 - Supernatant BL21 (DE3) [pFH772] – without IPTG
6 - SDS VII marker proteins
7 - Pellet BL21 (DE3) [pFH772] – without IPTG
correctly. However, the results show that the 64kDa protein was still in the pellet rather than in the supernatant suggesting the formation of inclusion bodies still (Figure 6.6).

The failure of added D,L2-CP to influence the solubility of DehR could be due to the D,L2-CP not being transported into the host cell in the presence of lactate. The mechanism of uptake of D,L2-CP into the cell was unknown but could possibly be via the lactate uptake system. If it is via the lactate system, the presence of lactate possibly inhibits the uptake of D,L2-CP into the bacterial cells due to the competitive effect of the two structurally similar compounds. The ability of the host cell to take up D,L2-CP was then investigated.

### 6.3.3 D,L2-CP uptake by BL21(DE3)
BL21(DE3) itself could not grow in minimal medium with D,L2-CP as a carbon source. To see whether BL21(DE3) could take up D,L2-CP, pSC3\[dehD^{+}\] and pSC4\[dehL^{+}\] were transformed into BL21(DE3). The transformants were inoculated into 20mM D,L2-CP minimal medium with and without 0.3mM IPTG. The results showed growth only in the presence of IPTG and the doubling time was 9 hours (Figure 6.7). DehD and DehL activity were detected in cell-free extracts with specific activities of 0.90 and 3.12\(\mu\)molCl\(^{-}\)/min/mg protein using D-2CP and L-2CP as a substrate, respectively (Table 6.1). Therefore, the ability of BL21(DE3) to take up D,L2-CP was confirmed.

### 6.3.4 D,L2-CP uptake by BL21(DE3) in the presence of lactate
To see whether there was uptake of D,L2-CP into the cells in the presence of lactate BL21(DE3) transformed with pSC3\(dehD^{+}\) and pSC4\(dehL^{+}\) was inoculated into media with IPTG containing (i) 20mM D,L2-CP plus 20mM lactate; (ii) 20mM lactate only; (iii) 20mM D,L2-CP only. Growth was observed in all media with a doubling time when lactate was present of approximately 5 hours. The specific activities for each dehalogenase enzyme are shown in Table 6.2. The specific activity when D,L2-CP was present in the growth medium was higher than without D,L2-CP. Therefore, D,L2-CP seems to enter the cells in the presence of lactate.
Figure 6.6. SDS-PAGE showing production of DehR by BL21(DE3)[pFH772] (dehR') in 20mM lactate minimal medium with 20mM D,L2-CP

Lane:
1- SDS VII marker proteins
2- Supernatant BL21(DE3) [pFH772] – with 0.01mM IPTG
3- Pellet BL21(DE3) [pFH772] – with 0.01mM IPTG
Figure 6.7. Growth of *E. coli* BL21(DE3)*[pSC3]* and *E. coli* BL21(DE3)*[pSC4]* in 20mM D,L2-CP minimal medium

Table 6.1. Dehalogenase specific activity of crude extract using D- and L-2CP as a substrate for cells grown on D,L2-CP+IPTG

<table>
<thead>
<tr>
<th>Dehalogenase</th>
<th>Construct</th>
<th>Specific activity</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DehD</td>
<td>BL21(DE3)pSC3</td>
<td>0.90</td>
<td>0.90</td>
</tr>
<tr>
<td>DehL</td>
<td>BL21(DE3)pSC4</td>
<td>3.12</td>
<td>3.12</td>
</tr>
</tbody>
</table>

Table 6.2. Dehalogenase activity of cells grown on lactate with IPTG and the effect of D,L2-CP on dehalogenase production

(Specific activity given in μmolCl⁻/min/mg protein)

<table>
<thead>
<tr>
<th>Dehalogenase enzyme prepared from:</th>
<th>D,L2CP+Lactate+IPTG</th>
<th>Lactate+IPTG</th>
<th>D,L2-CP+IPTG</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL21(DE3)<em>[pSC3]</em>(<em>dehD</em>)</td>
<td>1.12</td>
<td>0.25</td>
<td>1.21</td>
</tr>
<tr>
<td>BL21(DE3)<em>[pSC4]</em>(<em>dehL</em>)</td>
<td>3.00</td>
<td>0.90</td>
<td>3.52</td>
</tr>
</tbody>
</table>
6.4 Expression of the regulator gene in pUC18 vector
As described in the preceding section the DehR could not be produced in a soluble form using pT7-7 as vector. Accordingly, the dehR gene was cloned into another expression vector, pUC18, to see if soluble regulator protein could be produced using this vector.

6.4.1 PCR to generate BamHI and SalI restriction sites in a complete dehalogenase regulator gene for cloning into pUC18 vector
In order to get a full dehR gene PCR methodology was used. Sequence analysis indicated that the regulator gene did not contain BamHI and SalI sites (Appendix III). Primers were designed that would introduce these two restriction endonuclease sites as described (Material and Methods section 2.14, Table 2.4).

The primer for the 5’ end incorporated a BamHI site and a stop codon upstream of the start of the dehR gene. The stop codon was to prevent a lac fusion protein with DehR being made by the process of transcription and translation from the vector promoter. The primer at the 3’end was the same primer as described before (Materials and Method, Table 2.3) for incorporating a SalI site. The source of the DNA template used was a 4kb to 5kb SalI digested chromosomal DNA from Rhizobium sp.

The PCR product of 1.7kb was purified from a gel and the DNA was digested with BamHI and SalI before ligating into pUC18 digested with the same restriction endonucleases. The ligated DNA was transformed into E.coli NM522. The transformants were checked by preparing plasmid DNA and digesting it with the same restriction enzymes (Figure 6.8). The DNA construct was also checked by sequencing. The results show an identical sequence to the original sequence of dehR gene (data not shown). Such a construct was termed pFH18.

6.4.2 Growth and induction of NM522[pFH18] (dehR*) in LB/Amp
Cells were inoculated into a 100ml LB/amp medium and incubated at 37°C. IPTG (final concentration of 0.3mM) was then added at A_{680nm} of 0.5. The cells were then further incubated at the same temperature for another four hours.
Figure 6.8. pFH18 digested with \textit{BamHI}/\textit{SalI} showing 1.7kb DNA insert and 2.7kb vector

Lane:

1. 1kb ladder
2. pFH18 digested with \textit{BamHI}/\textit{SalI}
The cells were harvested, crude extract was prepared, and both supernatant and the resuspended cell pellet were analysed by SDS-PAGE. The results showed a protein band of 64kDa in the pellet rather than in the supernatant (Figure 6.9).

Induction at room temperature (21°C) was also tried in an attempt to avoid inclusion body formation. However, the 64kDa protein was still in the pellet rather than in the supernatant, suggesting that the formation of inclusion bodies still occurred (data not included).

### 6.4.3 Growth and induction of NM522 [pFH18] (dehR<sup>+</sup>) in minimal medium

To allow slower growth, NM522 [pFH18] was grown in 20mM lactate minimal medium. Cells from an overnight LB/amp culture (2ml) were washed twice with sterile minimal medium in order to get rid of the LB. They were then inoculated into 100ml lactate minimal medium and incubated at 37°C. IPTG (final concentration of 0.3mM) was added at A<sub>680nm</sub> of 0.5. The cells were further incubated for another four hours in the same temperature.

The cells were harvested and the fractionated crude extract was analysed. The results show there was no DehR protein present in the supernatant. However, the expected protein was observed in the pellet (Figure 6.10). Induction at room temperature (21°C) was also tested but gave a similar outcome.

Further attempts to improve the solubility of DehR protein, by growing cells in medium containing lactate but in the presence of 20mM D,L2-CP showed the expected protein was still in the pellet rather than in the supernatant. Therefore, the DehR protein remained insoluble. This suggests the presence of D,L2-CP had no effect on DehR solubility. The ability of NM522 to take up D,L2-CP was shown previously (section 3.2.1, Table 3.1). However, whether lactate prevent D,L2-CP uptake into the cells was then checked.
Figure 6.9. SDS-PAGE showing production of DehR protein from *E. coli* NM522 [pFH18] grown in LB/Amp

Approximately 5μg protein was loaded into each well

Lane:
1- SDS VII marker proteins
2- Supernatant NM522 [pFH18] – induced with 0.3mM IPTG at 37°C
3- Pellet NM522 [pFH18] – induced with 0.3 mM IPTG at 37°C
Figure 6.10 SDS-PAGE showing production of DehR protein from *E. coli* NM522 [pFH18] grown in lactate minimal medium

Lane:

1- SDS VII marker proteins
2- Supernatant NM522 [pFH18] – induced with 0.3 mM IPTG at 37°C
3- Supernatant NM522 [pFH18] – induced with 0.3 mM IPTG at 37°C (with 20 mM D,L2-CP)
4- SDS VII marker proteins
5- Pellet NM522 [pFH18] – induced with 0.3 mM IPTG at 37°C
6- Pellet NM522 [pFH18] – induced with 0.3 mM IPTG at 37°C (with 20 mM D,L2-CP)
6.4.4 D,L2-CP uptake by \textit{E.coli} NM522 in the presence of lactate

To see whether there was uptake of D,L2-CP into the cells in the presence of lactate, NM522 \( [pSC3](dehD^+) \) and NM522 \( [pSC4](dehL^+) \) were grown in (i) 20mM lactate minimal medium plus 20mM D,L2-CP; (ii) 20mM lactate only; and (iii) 20mM D,L2-CP only, all with 0.3mM IPTG. Crude extracts were prepared from each growth condition and the dehalogenase specific activities are shown in Table 6.3.

The specific activity with D,L2-CP present in growth medium was higher compared to growth without D,L2-CP. Therefore, D,L-2CP seems to enter the cells in the presence of lactate.

Table 6.3. Dehalogenase activity of cells grown on lactate with IPTG and the effect of D,L2-CP on dehalogenase activity (Specific activity given in \( \mu \text{molCl}^-/\text{min/mg protein} \))

<table>
<thead>
<tr>
<th>Dehalogenase enzyme prepared from:</th>
<th>Growth condition:</th>
<th>D,L2CP+Lactate+IPTG</th>
<th>Lactate+IPTG</th>
<th>D,L2-CP+IPTG</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM522(<a href="dehD%5E+">pSC3</a>)</td>
<td></td>
<td>1.12</td>
<td>0.23</td>
<td>1.21</td>
</tr>
<tr>
<td>NM522(<a href="dehL%5E+">pSC4</a>)</td>
<td></td>
<td>4.00</td>
<td>0.92</td>
<td>4.32</td>
</tr>
</tbody>
</table>

6.5 Expression of the regulator gene in pET-32 Xa/LIC (Ligation Independent Cloning) vector

Attempts at producing an active soluble DehR protein by slower growth at lower temperature or in different vector such as pUC18 were not successful. Another alternative was to ligate the \( dehR \) gene into a Xa/LIC vector which is designed for cloning high level expression of the ligated genes fused to the 109 amino acid thioredoxin (11,675 Da) protein. Many proteins that are normally produced in an insoluble form in the \textit{E.coli} cytoplasm tend to become more soluble when fused with thioredoxin protein (LaVallie \textit{et al.}, 1993). The thioredoxin protein carries with it a specific site for proteolytic digestion. This allows the unwanted protein to be removed from the target protein.
6.5.1 Engineering of the regulator gene into the pET-32 Xa/LIC vector

The gene of interest was PCR amplified using primers from 5' and 3' ends to introduce complementary overhangs to the LIC vector. The template used was pFH772 (dehR*), which contained a complete sequence of dehR gene. The primers designed were described in detail in Materials and Methods (section 2.15, Figure 2.2a,b). The PCR product seen in Figure 6.11 shows the expected size of approximately 1.7kb. The PCR product was then purified from the gel followed by treatment with T4 DNA polymerase in order to generate the specific vector compatible overhangs. Annealing in this way was described as a very efficient way of ligating into the vector. The ligated plasmid was designated as pFHXa/LIC1 (dehR*).

6.5.2 Transformation and expression of pFHXa/LIC1 (dehR*) in BL21(DE3)

The ligated plasmid was transformed into E.coli in order to express the dehR gene. To allow slower growth, BL21(DE3) with transformed plasmid was grown in minimal medium in the presence of 20mM glycerol as carbon source at 30°C up to A_{680nm} reading of 0.3 to 0.4. IPTG (final concentration of 0.01mM) was then added and the cells incubated at the same temperature for 4 hours. On different occasions, cells were induced with IPTG at A_{680nm} reading of 0.3 to 0.4 and further incubated at room temperature (21°C) overnight. A fractionated cell-free extract was prepared and the pellet was resuspended in the buffer and analysed on SDS-PAGE gel. An extract from a control BL21(DE3)pFH772(dehR*) was prepared and the fractionated extract was also run on the same SDS-PAGE gel.

The results shown in Figure 6.12 and Figure 6.13 suggest insoluble material was observed associated with inclusion bodies. The size of the target protein was slightly bigger (approximately 74kDa) than the one derived from pFH772 (dehR*) (64kDa) due to the thioredoxin protein (Figure 6.12). Cells grown at 21°C produced less DehR insoluble protein in the pellet but there was still none in the supernatant (Figure 6.13).
Figure 6.11. Agarose gel showing a 1.7kb PCR product for cloning into pET-32Xa/LIC

Lane:
1: 1kb DNA ladder
2: 1.7kb PCR product with generated overhangs to ligate into pET-32 Xa/LIC vector
3: Control without template DNA
4: Control without one of the primers
5: 1kb DNA ladder
Figure 6.12. SDS-PAGE analysis showing a DehR-thioredoxin fusion protein (74kDa) induced at 30°C

Approximately 10μg protein loaded into each well

Lane:
1: Protein markers
2: Supernatant of BL21(DE3)[pFHx/LIC1] (dehR<sup>+</sup>) grown with 0.01mM IPTG
3: Resuspended pellet of BL21(DE3)[pFHx/LIC1] (dehR<sup>+</sup>) grown with 0.01mM IPTG
4: Resuspended pellet from BL21(DE3)[pFH772] (dehR<sup>+</sup>)
5: Protein markers
Figure 6.13. SDS-PAGE analysis showing a DehR–thioredoxin fusion protein (74kDa) induced at 21°C
Approximately 20μg protein loaded into each well
Lane:
1: Protein markers
2: Supernatant of BL21(DE3)[pFHXa/LIC1] (dehR<sup>+</sup>) grown with 0.01mM IPTG
3: Resuspended pellet of BL21(DE3)[pFHXa/LIC1] (dehR<sup>+</sup>) grown with 0.01mM IPTG
4: Resuspended pellet from BL21(DE3)[pFH772] (dehR<sup>+</sup>)
5: Protein markers
None of the experiments that had been carried out using different vectors for dehR gave any significant amount of recombinant protein in the soluble fraction. Accordingly, it was decided to attempt to develop a strategy of re-folding the protein from the inclusion bodies.

### 6.6 Solubilisation of DehR protein from inclusion bodies

A high level of expression of heterologous proteins in E.coli often results in inclusion body formation. To obtain soluble, active protein the inclusion bodies must be solubilised and then re-folded. Each protein requires a different procedure, which must be determined empirically. There are various conditions to solubilise the inclusion bodies. In an initial experiment to solubilise the inclusion bodies 8M urea was used.

#### 6.6.1 Solubilisation and re-folding

The cell pellet from 100 ml culture E.coli BL21(DE3)[pFH772] (dehR*) grown in LB/amp was resuspended in 4ml of 0.1M Tris-acetate pH7.6 buffer followed by ultrasonication. The inclusion bodies were sedimented by centrifugation at 15,000g for 15 min at 4°C. The pellet was then resuspended in the 0.1M Tris-acetate pH7.6 buffer solution. An appropriate amount of suspended pellet (0.8mg protein) was then added into 0.1M Tris-acetate pH7.6 buffer plus 8M urea and left at room temperature (21°C) for one hour. Insoluble material was removed by centrifugation at 15,000g for 15 minutes. To allow refolding, the solubilized protein was diluted 1 in 10 into folding buffer (0.1M Tris-acetate pH7.6) to a final protein concentration of approximately 0.1mg/ml followed by incubation at room temperature for 1 hour. Finally, the solution was centrifuged and the supernatant and the pellet were analysed on an SDS-PAGE gel. The result shows that the protein was still present in the pellet (Figure 6.14).

From this first attempt, it was not possible to solubilise the DehR from inclusion bodies. Different conditions might be needed to solubilise the inclusion bodies using guanidineHCl(5-8M) or SDS and other refolding methods may be required. However, for some proteins it could be the most difficult process. Therefore, alternate ways of studying the DehR were needed. This will be described in the next section.
Figure 6.14. SDS-PAGE showing the attempt to solubilise the inclusion body protein

Lane:

1: Protein markers
2: Supernatant from urea treated sample
3: Pellet from urea treated sample
6.7 In vivo studies of Rhizobium sp. dehalogenase regulator gene function in E.coli

Production in vitro of soluble dehalogenase regulator protein from pFH772 (dehR⁺) was unsuccessful. Another option to investigate the function of the regulator gene was to make a construct consisting of a dehalogenase structural gene and the regulator gene together for in vivo analysis.

The following section will describe cloning the dehR gene with the dehE gene and the dehR gene with the dehD and dehL genes. Then the effect of the regulator gene product on expression of these structural genes can be investigated in the E.coli host.

6.8 Production of dehE and dehR co-construct (pHC2)

To make a construct with dehE and dehR in the same vector, DNA from three plasmids, pFH648, pFH45 and pFH100 was required. Plasmid pFH648 that carried the dehE gene was digested with SalI. The desired fragment of approximately 1.5kb carrying dehE was purified from an agarose gel and ligated into SalI digested pFH45 (incomplete dehR⁺). The ligated fragment was then transformed into an E.coli host. Plasmid DNA from transformants was then prepared.

To determine whether the SalI fragment had been incorporated the plasmid DNA was digested with SalI. Further digests with SacI indicated that this plasmid had the desired orientation where the transcription of the dehE gene was in the opposite direction to that from the vector promoter. This construct was designated as pHCl (incomplete dehR⁺ and dehE⁺) (Figure 6.15).

pFH100 carries the sequence that completes the dehR gene as a HindIII fragment. This fragment was ligated into pHCl digested with HindIII and transformed into competent E.coli. A number of transformants were selected and plasmid DNA prepared. The presence of the 1kb HindIII fragment in pHCl was confirmed by HindIII digests. The required orientation of the insert was shown to be present by PstI digestion. The final construct termed pHC2 (dehR⁺ and dehE⁺), is shown in Figure 6.16 with the structural gene direction of transcription being opposite to that of the vector promoter, as desired.
Figure 6.15. Construction of pHCl(partial $dehR^+$, $dehE^+$)

SalI fragment (1.5kb) of pFH648 was inserted into pFH45 to give pHCl

Key to enzymes: E=EcoRI, Sa=SacI, S=SalI, X=XhoI, H=HindIII.

Arrows indicate the direction of transcription of $dehE$ and $dehR$ genes.

Multicloning region shown as hatched (not to scale)
Figure 6.16. A restriction enzyme site map of pHC2 (dehR+, dehE+)

HindIII fragment (1kb) from pFH100 ligated into pHCl to produce pHC2.

Key to enzymes: E=EcoRI; P= PstI; Sa=SacI; S=SalI; X=XhoI; H=HindIII; Sp=SphI
6.8.1 Expression of dehE in NM522 [pHC2](dehR+, dehE+)

E.coli NM522 could not grow on D,L2-CP as a carbon and energy source without expression of a dehalogenase gene. To see whether the regulator gene controls the expression of the dehE gene dehalogenase production was monitored by checking growth on D,L2-CP. To allow this to happen, the dehR gene product needs to be produced and expression of this gene relies on the E.coli host RNA polymerase. It is necessary for the host polymerase to recognise the putative dehR promoter so that the regulator protein could be made. This experiment was expected to work provided that promoters for both genes could be recognised by the RNA polymerase of the host organism.

To prepare the inoculum, NM522[pHC2] were grown overnight in 20mM lactate minimal medium plus ampicillin (100μg/ml) at 30°C. The culture (5ml) was centrifuged, the cells washed and used to inoculate 20mM D,L2-CP minimal medium (initial reading at A$_{680nm}$ was approximately 0.10). An internal control was set up with the same amount of cells inoculated into 100ml minimal medium with lactate as a carbon and energy source.

Figure 6.17(a) shows that NM522[pHC2] did not grow on D,L2-CP minimal medium to any significant extent over an 8 day period. This suggests NM522[pHC2] did not express the dehE gene. Figure 6.17(b), shows growth occurred on lactate with a doubling time of approximately 4 hours indicating that the inoculum contained viable cells. The uptake of D,L2-CP by NM522 was not a problem because with pSC3(dehD+) or pSC4(dehL+) transformed into that host cell it could grow on 20mM D,L2-CP minimal medium as long as IPTG was present to induce the dehalogenase gene (Chapter 3, section 3.2.1).

As a further check for any dehalogenase activity being expressed NM522[pHC2] was grown in glycerol minimal medium plus 10mM D,L2-CP as inducer. Growth was monitored at A$_{680nm}$ and the cells were harvested at late logarithmic phase. The cell-free extract was prepared and DehE was assayed with 2,2DCP and D,L2-CP as substrates. There was no dehalogenase enzyme activity detected. Since D,L2-CP uptake into the host cell is not a problem this outcome is possibly due to the lack of the
Figure 6.17(a). Measurement of BL21(DE3)[pHC2] (deh\(^R\), deh\(^E\)) growth in 20mM D,L2-CP minimal medium

Figure 6.17(b). Measurement of BL21(DE3)[pHC2] (deh\(^R\), deh\(^E\)) growth in 20mM lactate minimal medium
regulator gene product not permitting dehE gene expression. To check this, the fractionated cell-free extract was analysed further by SDS-PAGE to see whether DehR protein was present. The results show that the 64kDa DehR protein was difficult to distinguish from the total cellular protein and presumably not present. In addition the 64kDa protein was also not present in the insoluble form (data not included). However, results by SDS-PAGE can be confirmed by Western blotting provided that a specific antibody for DehR protein is made.

6.9 Production of a dehD/dehL and dehR co-construct

It was possible that pHCl (dehR+, dehE+) did not allow growth of NM522 on D,L2-CP minimal medium due to the failure of the E.coli RNA polymerase to transcribe the dehR gene. A new construct was designed in which expression of the dehR gene was controlled from the vector promoter. To make this construct pH772 (dehR+) as a dehR gene expression plasmid and a suitable dehD and dehL containing fragment were needed.

6.9.1 Production of dehD/L genes by PCR to incorporate into pH772(dehR+)

Using PCR a 6.5kb DNA fragment was amplified from pSC1 (Figure 6.18a) incorporating a Sall restriction site at the 3’ end. The PCR product was digested with Sall to give an approximately 5kb fragment for ligating into pH772.

The ligated Sall fragment can be incorporated in two different orientations. The desired orientation of the structural genes was with their expression in the opposite direction to that from the vector promoter. The restriction enzyme analysis confirmed the desired location of the inserted PCR product. This plasmid, designated pHC773 (dehR+, dehD+, dehL+), is described in Figure 6.18(b).

6.9.2 Control of the expression of dehD and dehL by dehR

BL21(DE3) [pHC773] (dehR+, dehD+, dehL+) was inoculated into minimal medium containing (i) 20mM D,L2-CP plus 0.01mM IPTG; (ii) 20mM D,L2-CP without IPTG and (iii) a control supplied with 20mM lactate to check the source of inoculum used was viable. In Figure 6.19 growth on D,L2-CP minimal medium can be seen, which
Figure 6.18(a). PCR of *dehD* and *dehL* using pSC1 as template DNA

Standard reverse (5'caggaaacagctatgacc3') and forward (5'attacgcgtggttgtaaa3') primers are shown on the left and right hand side (arrows).

Arrows also indicate direction of transcription of *dehD* and *dehL* genes

H: HindIII; E: EcoRI; S: SalI; P: PstI; Hi: HindII

Hatched: polycloning region

Figure 6.18(b). Restriction enzyme site map of pHC773(*dehR*+, *dehD*−, *dehL*+)

Arrows indicate direction of transcription of the genes

N: Ndel; Sa: SacI; H: HindIII; E: EcoRI; S: SalI; P: PstI; Hi: HindII

Hatched: polycloning region
Figure 6.19. Measurement of BL21(DE3)[pHC773] (dehR⁺, dehD⁺ dehL⁺) growth in 20mM D,L2-CP minimal medium with and without IPTG.
suggests dehD and dehL were expressed. Only a trace of growth was seen without added IPTG. This slight growth could be due to the presence of contaminants such as lactate in the D,L2-CP rather than due to dehalogenase action. This was confirmed when another control experiment using BL21(DE3) pFH772 (dehR*) showed the same slight growth in D,L2-CP minimal medium (Figure 6.21a). There was a long lag phase of about 2 days before the cells started to grow but eventually a maximum growth of A\textsubscript{680nm} 0.45 was achieved. The experiment was repeated twice to check the reproducibility of the results and the outcome was identical.

In an attempt to avoid the long lag phase the experiment was repeated with the initial reading A\textsubscript{680nm} increased up to 0.1. However, a similar lag phase was observed. This eliminates the possibility that the lag was an experimental error due to the low starting A\textsubscript{680nm} reading. The long lag phase presumably represents the time required for the organism to synthesise dehalogenase to support growth on D,L2-CP.

Dehalogenase activity was also checked by making cell-free extracts. Cells were harvested close to the end of growth (A\textsubscript{680nm} 0.4). The specific activity obtained is shown in Table 6.4. The extract did not show any activity with 2,2DCP, as expected, since only dehL and dehD were involved, but did show that both DehD and DehL were produced.

\textbf{Table 6.4 Specific activity for DehD/DehL from cells prepared from BL21(DE3)pHC773(dehR*, dehD*, dehL*)}

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity (µmolCl/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D,L-2CP</td>
<td>0.18</td>
</tr>
<tr>
<td>L-2CP</td>
<td>0.21</td>
</tr>
<tr>
<td>D-2CP</td>
<td>0.15</td>
</tr>
<tr>
<td>2,2DCP</td>
<td>0.00</td>
</tr>
</tbody>
</table>
A separate experiment was carried out to show the ability of pHC773 to produce DehR protein similar to the pFH772 (dehR+) construct. BL21(DE3) pHC773 (dehR+, dehD+, dehL+) was grown in LB/amp medium and induced with 0.01mM IPTG at A680nm reading 0.4. The cells were further incubated at 30°C for another 4 hours. Figure 6.20 shows DehR (64kDa protein) was observed as an insoluble form in the pellet of the fractionated sample suggesting that under these growth conditions the protein was found in inclusion bodies like pFH772(dehR+).

### 6.9.3 D,L2-CP requirement for dehD and dehL expression in BL21(DE3)pHC773(dehR+, dehD+, dehL+)

The investigation was extended to show that D,L2-CP is needed for the expression of the structural genes. For this BL21(DE3) [pHC773] were grown in lactate minimal medium plus IPTG with and without 10mM D,L2-CP. Cell-free extracts were prepared from both lots of cells to check for dehalogenase activity. A specific activity of 0.05μmolCl⁻/min/mg protein was found using D,L2CP as a substrate for the extract from growth on lactate with D,L2-CP present. An extract prepared from cells grown without D,L2-CP did not show any dehalogenase activity. This suggests D,L2-CP was needed for dehalogenase gene expression.

### 6.9.4 Structural gene requirement for growth of BL21(DE3)[pFH772] (dehR+) in D,L2-CP minimal medium

This experiment was designed to show that the dehalogenase structural genes are needed for growth in D,L2-CP minimal medium. BL21(DE3)[pFH772](dehR+) was inoculated into minimal media supplied with 20mM D,L2-CP plus 0.01mM IPTG and into 20mM lactate as a control.

Figure 6.21(a) shows that growth was not observed in the absence of the dehalogenase structural genes. The slight increase in A680nm reading was presumed to be due to impurities in D,L2-CP medium. Crude cell extract was prepared and no dehalogenase activity was detected. The control supplied with lactate showed growth indicating that the inoculum used was viable, Figure 6.21(b).
Figure 6.20. SDS-PAGE analysis showing production DehR from BL21(DE3)[pHC773](dehR⁺, dehD⁺, dehL⁻) grown in LB/amp

Lane:
1: Protein markers
2: Supernatant BL21(DE3)[pHC773]
3: Pellet BL21(DE3)[pHC773]
4: Control: DehR protein from pFH772 (dehR⁺) (pellet)
Figure 6.21(a). Measurement of BL21(DE3)pFH772(dehR') growth in 20mM D,L2-CP minimal medium supplied with IPTG

Figure 6.21(b). Measurement of BL21(DE3)pFH772(dehR') growth in 20mM lactate minimal medium
6.9.5 Dehalogenase regulator gene requirement for growth of BL21(DE3) pHC773(dehR*, dehD+, dehL*) in D,L2-CP minimal medium

6.9.5.1 Deletion of the regulator gene in pHC773 (dehR*, dehD+, dehL*)

To further investigate the function of the dehR gene to show that it was involved in dehalogenase gene expression a clone with dehR largely deleted was produced. Based on the restriction enzyme mapping of pHC773 (dehR*, dehD+, dehL*) (Figure 6.18b), it was possible to use EcoRI to delete over 75% of the dehR gene.

The desired fragment isolated from an EcoRI digest was re-ligated and transformed into BL21(DE3). The transformants were then checked by EcoRI digestion (Figure 6.22a). The plasmid with a truncated dehR gene was designated as pHC773-AR (dehD*, dehL*, ΔdehR*) (Figure 6.22b).

6.9.5.2 Expression of dehD and dehL in BL21(DE3)[pHC773-AR]

(dehD+, dehL+, ΔdehR*)

BL21(DE3)[pHC773-AR](dehD+, dehL+, ΔdehR*) was inoculated into D,L2-CP minimal medium plus IPTG. As a control the same amount of cells was inoculated into lactate minimal medium to check for viability. The results in Figure 6.23(a) show BL21(DE3)[pHC773-AR] did not grow on D,L2-CP minimal medium with IPTG during a 7 day period. The control experiment (Figure 6.23b) shows that the inoculum was viable. This suggests that the regulator gene product is required for the structural genes to be expressed and allow growth on D,L2-CP.
Figure 6.22(a). Agarose gel showing restriction enzyme digest of pH773-ΔR

EcoRI fragment (1.6kb) in pH773-ΔR (Lane 1) was absent compared to the parental plasmid pH773 (Lane 3).

Lane:
1 - pH773-ΔR / EcoRI
2 - 1kb DNA ladder
3 - pH773 / EcoRI

Figure 6.22(b). Restriction enzyme map of pH773- ΔR
Figure 6.23(a). Measurement of BL21(DE3)[pHC773-ΔR] growth in 20mM D,L2-CP minimal medium

Figure 6.23(b). Measurement of BL21(DE3)[pHC773-ΔR] growth in 20mM lactate minimal medium
6.9.6 Production of promoter-less \textit{dehD/dehL}

\textit{pHC773} (\textit{dehR}^\ast, \textit{dehD}^\ast, \textit{dehL}^\ast) included 1.2kb of sequence upstream of \textit{dehD} presumably carrying the putative promoter region. The PCR technique was used to generate a new DNA sequence from which the putative promoter region was deleted. A standard reverse primer was designed incorporating a \textit{SalI} site 17bp from the start of \textit{dehD} and the standard forward primer also incorporated \textit{SalI} from the 3' region. Both primers were used to copy pSC1 plasmid as shown in Figure 6.24(a) to generate the desired promoter-less 3.5kb PCR product.

The PCR product was digested with \textit{SalI} enzyme before ligating into \textit{SalI} digested \textit{pFH772} (\textit{dehR}^\ast). Restriction analysis was used to identify a construct in which the expression of the cloned \textit{dehD/dehL} genes was in the opposite direction to that from the vector promoter. This construct was designated as \textit{pHC773-\Delta P} (Figure 6.24b).

6.9.6.1 Sequencing of \textit{pHC773-\Delta P} (\textit{dehR}^\ast, \textit{dehD}^\ast, \textit{dehL}^\ast)

Before attempting any experiment, \textit{pHC773-\Delta P} was sequenced to be sure there is no mutation introduced by PCR. The full nucleotide sequence of the \textit{pHC773-\Delta P} was compared to the published sequence of \textit{dehD} and \textit{dehL} and showed that the sequences matched.

6.9.6.2 Expression of \textit{dehD/dehL} genes in \textit{BL21(DE3)} [\textit{pHC773-\Delta P}]

(\textit{dehR}^\ast, \textit{dehD}^\ast, \textit{dehL}^\ast)

A growth experiment was carried out with 20mM D,L2-CP minimal medium to see whether there is any effect of deleting the putative promoter region on \textit{dehD} and \textit{dehL} expression. Growth of \textit{BL21(DE3)}[\textit{pHC773-\Delta P}] was investigated with and without the IPTG necessary for \textit{dehR} expression.

Cells were incubated in three flasks that contained: (i) 20mM lactate; (ii) 20mM D,L2-CP plus 0.01mM IPTG; (iii) 20mM D,L2-CP without IPTG. Figure 6.25(a) shows that growth was not seen for \textit{BL21(DE3)}[\textit{pHC773-\Delta P}] on D,L2-CP with or without IPTG over a 7 day period. The control flask with lactate grew overnight, Figure 6.25(b). This experiment indicates that the putative promoter region is needed for structural gene expression and also suggests that there is only one promoter that controls both \textit{dehD} and \textit{dehL}.
A Primer with Sall site binding close to the dehD gene

Figure 6.24(a). Production of putative promotor-less sequence by PCR using pSC1 as the template DNA

Primers indicated as arrows: Reverse primer (5’attacgcgtcgaactttcaattaa3’) and forward primer (5’attacgcgtcgaegotgtaa3’)
H: HindIII; E: EcoRI; S: Sall; P: PstI ; Hi: HinCII
Hatched: polycloning region

Figure 6.24(b). Restriction enzyme site map of pH773-AP

The structural gene expression is in the opposite direction to avoid expression from the vector promoter. The orientation was confirmed by restriction enzyme digestion of the plasmid DNA
N: Ndel; Sa: SacI; H: HindIII; E: EcoRI; S: Sall; P: PstI ; Hi: HinCII
Figure 6.25(a). Measurement of BL21(DE3)[pHC773-ΔP] growth in 20mM D,L2-CP minimal medium with and without IPTG

Figure 6.25(b). Measurement of BL21(DE3)[pHC773-ΔP] growth in 20mM lactate minimal medium
6.10 Investigation of the role of DehR in dehE expression

The previous section indicated that the dehR gene controlled expression of both dehD and dehL. However, it had been proposed by Leigh (1986) that the same regulator gene controls expression of the dehE gene. Experiments designed to test this proposal are described in this section.

6.10.1 Ligation of the SalI fragment from pSC520 carrying the dehE gene into pFH772 (dehR*)

A SalI fragment from pSC520 containing the dehE gene was cloned into SalI digested pFH772 (dehR*), Figure 6.26(a). The distance from SalI to the start of dehE in pSC520 was 76bp and this may or may not include the promoter region.

The desired orientation of the dehE gene was that which avoided its transcription from the vector promoter. The ligation of the SalI fragment in the required orientation was confirmed by restriction analysis as shown in Figure 6.26(b). EcoRI/BamHI digests gave two bands of the required size 3.2kb and 2.6kb to indicate the desired orientation (Lane 3 in Figure 6.26b). The plasmid was then designated pHC774 (dehR+, dehE*).

6.10.2 Expression of the dehE gene in BL21(DE3)[pHC774] (dehR+, dehE*)

BL21(DE3)[pHC774] (dehR+, dehE*) was inoculated into 20mM D,L2-CP minimal medium with and without 0.01mM IPTG and growth was monitored at 30°C over a period of time. A control experiment using 20mM lactate minimal medium plus ampicillin was set up at the same time to make sure that the inoculum used was viable.

The results from this experiment showed that growth was not seen in the D,L2CP media with or without IPTG over eight days (Figure 6.27a) although growth on 20mM lactate was observed readily (Figure 6.27b).

Growth of BL21(DE3)[pHC774] (dehR+, dehE*) in D,L2CP plus lactate with and without IPTG was carried out. Cell-free extract was prepared and used in enzyme assays. The results did not show any chloride release suggesting that dehalogenase enzyme was not present.
Sall fragment (approximately 1.4kb in size) to be inserted into pFH772

Figure 6.26(a). *Sall* fragment from pSC520 inserted into pFH772 to produce pHC774 (*dehR*, *dehE*)

Arrows: direction of transcription; N: NdeI; E: EcoRI; S: SalI; B: BamHI

Lane:

1- lkb DNA ladder
2- pHC774 digested with *Sall*
3- pHC774 digested with EcoRI/BamHI

Figure 6.26(b). Restriction enzyme analysis of plasmid DNA to confirm the orientation of the *Sall* fragment containing the *dehE* gene

Lane:

1- 1kb DNA ladder
2- pHC774 digested with *Sall*
3- pHC774 digested with EcoRI/BamHI
Figure 6.27(a). Measurement of BL21(DE3)[pHC774] (dehR\(^+\), dehE\(^+\)) growth in D,L2-CP minimal medium with and without IPTG

Figure 6.27(b). Measurement of BL21(DE3)[pHC774] (dehR\(^+\), dehE\(^+\)) growth in 20mM lactate minimal medium
A separate experiment was carried out to show the ability of BL21 (DE3)[pHC774] \((dehR^+ \text{, } dehE^+\)) to produce DehR protein similar to that of pHC773 (section 6.9.2). BL21 (DE3)[pHC774] \((dehR^+ \text{, } dehE^+)\) was grown in LB/amp medium and induced with 0.01mM IPTG as described in section 6.9.2. The fractionated sample was prepared and run on SDS-PAGE to confirm expression of \(dehR\) in BL21 (DE3)[pHC774] \((dehR^+ \text{, } dehE^+)\). The results show that DehR protein was present mainly as inclusion bodies (data not included). One possible reason for the failure to grow and produced dehalogenase enzyme was that the 76bp region between the \(SalI\) site to the start of \(dehE\) might not include the putative promoter sequence. However, if the complete putative promoter sequence is present in the construct another possibility might be that \(dehE\) is not regulated by the \(dehR\) gene although both genes are very close on the genome. The presence of a promoter can be checked by sequence comparison and will be described in the following section.

### 6.11 Identification of \(dehE\) and \(dehR\) putative promoters by sequence comparison

In *Pseudomonas putida* PP3 upstream of \(dehl\) was an intergenic region between \(dehl\) and the regulator gene \((dehR)\) where the promoters of the genes have been tentatively identified. Because of the similar situation to \(dehR\) and \(dehE\) in *Rhizobium sp.* it might be possible to identify the promoter by sequence comparison.

The intergenic region between \(dehR\) and \(dehE\) genes (292bp) from *Rhizobium sp.* was compared to the equivalent intergenic sequence from *Pseudomonas putida* PP3 (283bp) (Figure 6.28a). The result shows an overall 44% identity (Figure 6.28b). The putative -24/-12 and -35/-10 promoters proposed for *Pseudomonas putida* PP3 were identified in *Rhizobium sp.*, as shown in Figure 6.28b. The putative -24/-12 was found close to the \(SalI\) restriction enzyme site but not included in the pHC774 \((dehR^+ \text{, } dehE^+)\) construct. These -24/-12 and -35/-10 promoter regions were possibly involved in controlling \(dehE\) and \(dehR\) genes, respectively.

*Alcaligenes xylosoxidans* ssp. *denitrificans* ABIV (Bromkamp et al., 1997) was also reported to have D,L-haloalkanoic acid dehalogenase \((dhlIV)\) and regulator \((dhlRIV)\) genes similar to that of *Rhizobium sp.* and *Pseudomonas putida* PP3.
Figure 6.28(a). Intergenic region of Pseudomonas putida PP3 dehR and dehI (283bp) (Thomas, 1990). The putative -12/-24 promoter for dehI transcription identified conformed to the consensus -12(GC)/-24(GG) (Johnston and Downie, 1984; Ausubel, 1984; Kustu et al., 1989). The putative -10/-35 promoter for dehR was also identified. Letters in bold are the start codon for dehalogenase regulator and structural genes.

Figure 6.28(b). Intergenic region sequence comparison between Rhizobium sp. and Pseudomonas putida PP3.

The putative promoter regions of the Pseudomonas putida PP3 sequence are in bold and underlined. The promoter location of Rhizobium sp. is identified on the basis of sequence homology only. The conserved base pairs are indicated in bold at -24(GG) and -12(GC). The overall percent identity is 44%. The Rhizobium sp. dehalogenase regulator (dehR) and structural gene (dehE) start codons are in bold. Pseudomonas putida PP3 dehI structural gene start codon and dehR are also marked in bold.
However, the size of the intergenic region between \textit{dhlR}_{IV} and \textit{dhIV} of \textit{Alcaligenes xylosoxidans} (1.5kb) was 5x greater than for \textit{Rhizobium sp.} and \textit{Pseudomonas putida PP3} (Figure 6.29a). The intergenic region of \textit{Rhizobium sp.} and \textit{Alcaligenes ssp.} (between residues 351 and 671) was then compared to see if there was any matching promoter region between the two sequences. Based on the putative promoter region of \textit{Rhizobium sp.} that was determined by sequence comparison to that of \textit{Pseudomonas putida} PP3, the newly located putative promoter sequences of \textit{Alcaligenes ssp.} were proposed as marked in bold (Figure 6.29b). Since the percent identity is 45% similar to that of when intergenic sequence from \textit{Rhizobium sp.} was compared to the \textit{Pseudomonas putida} PP3, it was proposed that the intergenic region of \textit{Alcaligenes} is approximately 300bp and not 1.5kb. The remaining nucleotide residues from position 350 to position 1 (from Figure 6.29a) were translated into amino acid sequence. This sequence was then compared to the DehR from \textit{Rhizobium sp.}, but did not match. This suggests the absence in this region of a regulatory gene sequence so the size of the intergenic region in \textit{Alcaligenes ssp.} may well be 1.5kb as reported earlier (Brokamp \textit{et al.}, 1997) and the identified promoters (as proposed in Figure 6.29b) were not promoter regions.

A similar investigation was carried out by sequence comparison for \textit{Xanthobacter autotrophicus} GJ10 (van der Ploeg and Janssen, 1995) (Figure 6.30a). Within the intergenic region between nucleotide residues 108 and 115 the -24/-12 promoter was located. The intergenic sequence for \textit{Xanthobacter} was proposed to have a similar size to the \textit{Rhizobium sp.} intergenic region of approximately 300bp. For that reason the sequence from residue 251 to residue 554 was then compared to the \textit{Rhizobium} intergenic region as shown in Figure 6.30b. The sequence identity was 41%. A putative -24/-12 promoter sequence was seen almost similar to the putative -24/-12 of \textit{Rhizobium sp.}. The nucleotides from residue 250 to the start of the \textit{dhlR} (Figure 6.30a) were then translated into amino acid sequence and compared to the \textit{Rhizobium sp.} DehR amino acid sequence. The result did not show any sequence identity to DehR. This might suggest that the start of the \textit{dhlR} was as reported before (van der Ploeg and Janssen, 1995) and the indication of having promoter as proposed in Figure 6.30b was not a true promoter region.
Figure 6.29a. Intergenic sequence of \textit{dhllV} \textit{Alcaligenes ssp}: The putative $\sigma^{54}(-24 / -12)$ promoter consensus sequences are indicated in bold. 

S/D: Shine Dalgarno sequence (underline)

Start codon of the dehalogenase \textit{dhllV} structural gene in bold

Arrows indicate direction of transcription
Figure 6.29b. Sequence comparison of the intergenic region between *Rhizobium* sp. and *Alcaligenes* ssp. (Brokamp et al., 1997).

The proposed putative promoter regions of *Alcaligenes* ssp. sequence are in bold. Start codons of dehalogenase regulator (*dehR*) and structural gene (*dehE*) of *Rhizobium* sp. are in bold. The percent identity is 45%. Arrows indicate direction of transcription.
Figure 6.30a. Intergenic sequence of Xanthobacter autotrophicus GJ10 (van der Ploeg and Janssen, 1995)

The putative $\sigma^34 (-24/-12)$ promoter consensus sequence is underlined. Letters in bold indicate the start codon for the dehalogenase regulator (dhlR) and structural (dhlC) genes. Arrows indicate direction of transcription.
Figure 6.30(b). Sequence comparison of the intergenic region between *Rhizobium* sp. and *Xanthobacter autotrophicus* GJ10

The proposed putative promoter region are in bold: -24 (GG) and -12 (GC)

Percent identity is 41%. Arrows indicate direction of transcription.
6.12 Identification of *dehD* and *dehL* putative promoter sequence by sequence comparison

The 142bp upstream *dehD* and the intergenic region between *dehD* and *dehL* was then analysed to look for the putative promoter by sequence comparison to the intergenic region of *Pseudomonas putida* PP3 (Figure 6.31a and b). The results showed, no sequence that matched any -24/-12 or -10/-35 sequence. It was concluded that the 142bp upstream *dehD* did not contain a promoter region but there was no sequence available further upstream. In addition, the intergenic region between *dehD* and *dehL* also did not contain any promoter as expected from the previous experiment in section 6.9.6 using the promoter-less construct.

6.13 Discussion

Several experimental approaches were attempted in the current study to minimise formation of inclusion bodies and improve protein folding as described by Schein, 1991. These include the growth of bacterial cultures at lower temperature (Cabilly, 1989), cloning into a different vector such as pUC18 or cloning into pET-32Xa/LIC vector that allows formation of a fusion partner with *E.coli* thioredoxin protein (La Vallie *et al.*, 1993) but this still failed to prevent DehR being formed as inclusion bodies. Induction at the lower temperature had allowed DehE to be produced successfully in the soluble form in the pT7-7 system (Stringfellow *et al.*, 1997) but not DehR possibly because it is twice the size of *dehE*.

The formation of inclusion bodies does offer several advantages such as protection against host protease, simpler purification and also higher protein yields (Makrides, 1996; Baneyx and Georgiou, 1992; Nossal and Heppel, 1966).

So another option was to investigate ways to generate an active native protein by unfolding the inclusion bodies and re-folding the protein (Rudolf and Lilie, 1996). However, the preliminary investigations did not give satisfactory results. The investigation using inclusion bodies was not carried any further because an alternative way to characterise the DehR protein *in vitro* was developed.
Figure 6.31(a). Intergenic region sequence comparison between *Pseudomonas putida* PP3 and upstream sequence from *dehD* of *Rhizobium* sp.

Promoter region and start codon are in bold. (Percent identity: 40%)

Arrows indicate direction of transcription

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**Figure 6.31(b). Intergenic region sequence comparison between *Pseudomonas putida* PP3 and *dehD/dehL* of *Rhizobium* sp.

Promoter region and start codon are in bold. (Percent identity: 31%)

Arrows indicate direction of transcription
Leigh (1986) proposed that dehalogenase genes in *Rhizobium sp.* were positively regulated (Figure 1.5) with a promoter to control *dehE* and a different promoter controlling the *dehD* and *dehL* genes. In the present investigation, the function of DehR in controlling expression of the *dehD* and *dehL* structural genes was successfully tested. This was achieved by using a construct carrying structural genes and the regulator gene and monitoring dehalogenase expression in an heterologous host cell by growth on D,L2-CP. In addition to that a single promoter was seen controlling both *dehD* and *dehL* structural genes based on the expression of *dehD/dehL* genes in BL21(DE3) pHC773-ΔP (*dehR<sup>+</sup>, *dehD<sup>+</sup>, *dehL<sup>+</sup>). If there was a separate promoter in the intergenic region between *dehD* and *dehL*, expression of *dehL* gene will be independent of *dehD*. However, the absence of growth suggests that the same promoter is needed for the expression of both genes (Figure 6.25a). This experiment supported the model proposed by Leigh (1986) where a single promoter controlled both *dehD* and *dehL*.

Expression of *dehD* and *dehL* only occurred when transcription was triggered by the product of the adjacent gene, *dehR*, in pHC773 (*dehR<sup>+</sup>, *dehD<sup>+</sup>, *dehL<sup>+</sup>). The *dehR* transcription was initiated from a vector promoter. However, expression of *dehE* in pHC774 (*dehR<sup>+</sup>, *dehE<sup>+</sup>*) (with *dehR* transcription from a vector promoter) was not observed, presumably due to the lack of the promoter region for *dehE*. It was described earlier that based on sequence homology with the intergenic region of *Pseudomonas putida* PP3 a Rhizobial -24/-12 promoter for *dehE* was identified and this sequence was absent from the only construct that could be generated using the naturally occurring *SalI* site.

On the other hand pHC2 is a clone that includes the putative promoter region upstream of *dehE*. However, *E.coli* transformed with pHC2 (*dehR<sup>+</sup>, *dehE<sup>+</sup>*) did not grow on D,L2-CP possibly because the *dehR* gene product was not made in the host organism due to the putative promoter region upstream of *dehR* not being recognised by *E.coli* RNA polymerase. This was shown when the cell-free extract prepared from NM522 pHC2 (*dehR<sup>+</sup>, *dehE<sup>+</sup>*) run on the SDS-PAGE did not show a clear band that would indicate the presence of 64kDa DehR protein and none in the insoluble forms. However, this can always be confirmed by Western blot experiment provided that antibody for DehR was made.
In the present investigation, extensive studies were carried on *E. coli* BL21(DE3) transformed with pHCl773 (*dehR\(^+\), dehD\(^+\), dehL\(^+\)*) using growth on D,L2-CP as a measure of dehalogenase production to show different elements were necessary for *dehD* and *dehL* gene expression. The requirement for regulator gene product, a co-inducer and the promoter sequence were demonstrated clearly. This is the first investigation to show that all three elements were needed for dehalogenase gene expression. When one of the elements was absent, there was no expression of the structural genes.

The nucleotide sequence of *dehR* in *Pseudomonas putida* PP3 revealed sequence similarity (in both DNA and deduced protein sequences) to a number of other \(\sigma^{54}\) dependent activator proteins. A putative -24/-12 promoter was identified in the nucleotide sequence upstream of *dehl* by sequence comparison to other consensus sequence of the -24/-12 promoter. In *Rhizobium sp.* the same putative promoter was also located upstream of *dehE* and possibly required \(\sigma^{54}\)-dependent activator proteins for transcription. This possibly is the reason why pSC520 (*dehE\(^+\)*) did not express DehE very well in *E. coli*.

The dehalogenase enzyme regulation of *Xanthobacter autotrophicus* GJ10 is not well understood (van der Ploeg and Janssen, 1995). However, the sequence upstream of *dhlB*, the gene encoding haloalkanoic acid dehalogenase, was determined and showed ORFs that may function in transport of acids and regulation of expression of *dhlB*. The protein encoded by the putative dehalogenase regulator, *dhlR*, showed high similarity with proteins from the family of transcriptional activators which activate expression from -24/-12 promoters. Expression from this promoter requires the RNA polymerase factor \(\sigma^{54}\) and a transcriptional activator. Thus, the expression of the transport protein *dhlC* and possibly *dhlB* may be under the positive regulatory control of *dhlR* dependent on \(\sigma^{54}\) (van der Ploeg and Janssen, 1995). In *Pseudomonas putida* PP3 the *dehl* was also controlled by -24/-12 promoter as indicated by the lack of expression of *dehl* observed in an *rpoN* mutant of *Pseudomonas putida* PP3 (Thomas et al. 1992a). Generally, activation of \(\sigma^{54}\) dependent promoters occurs in response to a situation of environmental stress (Thöny and Hennecke, 1989).
In *Alcaligenes xylosoxidans* ssp. *denitrificans* ABIV there are some indications from restriction patterns and initial sequencing data that a gene encoding a putative $\sigma^{54}$-dependent activator, *dhlRIV*, similar to the *dehRI* regulatory gene from *Pseudomonas putida* PP3 was located upstream of *dhllV*, a gene encoding haloalkanoic acid dehalogenase. The encoded amino acid sequence of the haloalkanoic acid dehalogenase of *Alcaligenes xylosoxidans* ssp. *denitrificans* ABIV gave 70% identity to *Rhizobium* sp. DehE enzyme (Stringfellow *et al.*, 1997). However, the sequence of *dhlRIV* was not available to see the relationship with *Rhizobium* sp. *dehR* gene.

As described earlier expression of *dehE* was not observed in *E.coli* transformed with pHC774 (*dehE*, *dehR*) plasmid. The most likely reason is that the putative -24/-12 promoter region for the *dehE* gene was not included in the construct DNA. Although it could not be proven by the experiment, there was a strong suggestion that DehR controls expression of the *dehE* gene in *Rhizobium* sp. because the two genes were contiguous in the genome (Figure 6.32a). In *Pseudomonas putida* PP3 the location of *dehRI* is adjacent to *dehI* with the opposite direction of transcription (Figure 6.32b). That is exactly similar to the *dehR* and *dehE* genes in *Rhizobium* sp. Further support for this view was seen in *Alcaligenes xylosoxidans* ssp. *denitrificans* ABIV (Brokamp *et al.*, 1997) (Figure 6.32c) and *Xanthobacter autotrophicus* GJ10 (van der Ploeg and Janssen, 1995) (Figure 6.32d) where positive regulator genes were adjacent to a dehalogenase structural gene but with the opposite direction of transcription. However, in both these cases the only evidence to show the regulator gene product controlled the structural gene was the presence of the putative $\sigma^{54}$ (-12/-24) promoter consensus sequence identified in the upstream region of the structural genes (Brokamp *et al.*, 1997; van der Ploeg and Janssen, 1995).

Although putative regulator genes have been identified in *Pseudomonas putida* PP3, *Xanthobacter autotrophicus* GJ10 and *Alcaligenes xylosoxidans* ssp. *denitrificans* ABIV, the regulator gene product was not further studied except by sequence comparison. In addition the identified promoter upstream *dhll/dehRI* genes from *Pseudomonas putida* PP3 are highly speculative and require physical evidence by footprinting or gel shift experiment.
Figure 6.32(a) Map of pFH648 (dehE, dehR incomplete) showing the relationship between the 2-haloalkanoic acid dehalogenase gene dehE and its adjacent regulator gene, dehR (incomplete sequence). The arrows indicate the direction of transcription of both genes. The putative promoters for each gene are located within a control region designated as ‘p’ (0.29kb). Both genes are not to scale.

Figure 6.32(b) Map of part of the transposon DEH showing the relationship between the 2-haloalkanoic acid dehalogenase gene dehl and its adjacent regulator gene, dehR (Topping et al., 1995).

The arrows indicate the direction of transcription deduced from the promoter motifs located within a control region designated as ‘c’ (0.28kb). Both genes are not to scale.

Figure 6.32(c) Physical map of 2-haloalkanoic acid dehalogenase dhllV and its regulatory gene, dhlRIV of Alcaligenes xylosoxidans ssp. denitrificans ABIV (Brokamp et al., 1997).

The gap between dhllV and its regulatory gene (dhlRIV) (p region) is 1.5kb. Both genes are not to scale.

Figure 6.32(d) The location of dhlC/dhlB and dhlR of Xanthobacter autotrophicus GJ10 (van der Ploeg and Janssen, 1995).

The gap between the structural genes and the putative regulatory gene (p region), is about 0.55kb. Both genes are not to scale.
In the present study, the control of *dehE* by the same regulatory gene that controls *dehD* and *dehL* was suggested by physical location. However, in order to see if the same regulator gene controls expression of *dehE* the original *SalI* site (Figure 6.28b), needs to be mutated so that the putative −24/-12 promoter region can be included along with *dehE* in a *SalI* fragment made using PCR. In addition to that it is of interest to confirm the analysis by gel shift experiment to show a single regulator gene controls all three dehalogenases provided that the regulator gene product can be solubilised.
CHAPTER 7

GENERAL CONCLUSIONS

&

FUTURE WORK
7.1 General conclusions

Rhizobium sp. had been shown to synthesise three dehalogenases when grown on, or in the presence of certain halogenated aliphatic acids. As part of an attempt to understand why multiple dehalogenase proteins were produced, kinetic analysis (Km, Kcat and Specificity Constant) was undertaken.

The kinetic study showed that DehL and DehD were efficient enzymes than DehE (L-2CP=3.27x10⁴ M⁻¹ sec⁻¹; D-2CP=1.56x10⁴ M⁻¹ sec⁻¹) for L-2CP (1.33x10⁵ M⁻¹ sec⁻¹) and D-2CP (1.12x10⁵ M⁻¹ sec⁻¹) respectively, with significantly higher Specificity Constants values. In addition, both DehL and DehD had higher affinity for D,L2-CP as shown by low Km values compared to DehE.

The ability to utilise 2,2DCP as a sole carbon source was conferred only by DehE. Growth of Rhizobium sp. on low 2,2DCP concentration did not suggest any new dehalogenase(s) were produced. The Km value of DehE for 2,2DCP of 0.19mM was compatible with the observed growth at 0.2mM 2,2DCP concentration. However, during growth at low and high 2,2DCP concentration, DehL and DehD were also present although it was confirmed that both enzymes did not act on 2,2DCP. An interesting observation from experiments was the three dehalogenases were not co-ordinately controlled since the relative activity ratios of the three enzymes varied with substrate concentrations. Leigh (1986) using various co-inducers also showed similar phenomena that allowed him to suggest that regulation of Rhizobium sp. dehalogenase expression was complex.

A possible explanation for dehalogenase multiplicity is suggested by variation in the G+C content of the three dehalogenase genes. Conceivably, dehL and dehD might have originated in Rhizobium sp. whereas, dehE might have been obtained separately via plasmid transfer. Similar observation was seen where D,L-haloalkanoic acid dehalogenase (the dehalogenase enzyme equivalent to DehE in Rhizobium sp.) from Alcaligenes xylosoxidans ssp. (Brokamp et al., 1997) was incorporated later into the chromosomal DNA of the same organism by plasmid pFL40 found in Alcaligenes xylosoxidans ssp.
Only three substrates (2,2DCP, D,L2-CP and D,L2-BP) were used as sole source of carbon and energy for *Rhizobium sp.*, and the presence of more than one enzyme might be useful to act on un-investigated halogenated aliphatic acids. In addition, the precise mechanism(s) by which the three dehalogenases attack halogenated aliphatic acids is not known. Further investigation is needed to elucidate this.

The earlier investigations with mutants had suggested that all three dehalogenase genes were under the control of a single regulator gene. Cairns *et al.*, (1996) had shown that *dehD* and *dehL* were neighbouring genes. The cloning described here of *dehR* showed similar close arrangement of *dehE* and *dehR* which perhaps implied that *dehR* controlled expression of *dehE* although this could not be demonstrated in this work. However, the results here suggested that DehR did regulate expression of *dehD* and *dehL*. Further confirmation by physical evidence might be necessary.

7.2 Future work

7.2.1 Further characterisation of dehalogenase enzymes

*dehL*, *dehE* and *dehD* genes from *Rhizobium sp.* have been cloned and these proteins can be produced sufficiently well to allow large amounts to be made. As a result it is possible to investigate further their chemical and biological properties.

Crystal structures of the three dehalogenases may be obtained that can give information on the 3-dimensional structure of their active sites that may explain the limited substrate specificity of the *Rhizobium sp.* dehalogenases.

Chemical modification could also be used to gain information on amino acid(s) at the active site. This procedure will involve using chemical agents that will react specifically with amino acid to see whether activity is lost and the loss prevented by the presence of the substrate.

7.2.2 N-terminal amino acid sequencing of dehalogenase from growth at low 2,2DCP concentration

The identities of the dehalogenases in cells grown at low concentration of 2,2DCP can be further investigated by determination of their N-terminal amino acid sequences.
7.2.3 Dehalogenase gene organisation

Further studies can be carried out using the available cloned DNA (Figure 7.1. and Figure 7.2.) to investigate:

i) The location of the promoter region that controls both \textit{dehD} and \textit{dehL} genes in pSC1 (\textit{dehD}^+, \textit{dehL}^+). By obtaining the full sequence upstream of \textit{dehD} (Figure 7.1) the location of the putative promoter could be sought by sequence comparison to identify -24/-12 promoter or -10/-35 type promoter.

ii) At present, there is no information on how the halogenated substrate is transported into the \textit{Rhizobium sp..} The full DNA sequence of pSC1 (\textit{dehD}^+, \textit{dehL}^+) (Figure 7.1.) and pSC530 (\textit{dehE}^+, \textit{dehR}^+ incomplete) (Figure 7.2) may help to identify the putative permease gene by comparison to the proposed amino acid sequence from \textit{Xanthobacter autotrophicus GJ10} (van der Ploeg and Janssen, 1995). The location of putative transporter gene in \textit{Xanthobacter autotrophicus GJ10} was upstream of the 2-haloalkanoic acid dehalogenase (\textit{dhlB}) gene (van der Ploeg and Janssen, 1995).

7.2.4 Characterisation of dehalogenase regulator gene product

7.2.4.1 Solubilisation of the inclusion body dehalogenase regulator gene product

It was difficult to obtain a soluble active DehR protein from the heterologous expression in \textit{E.coli}. Various ways were suggested in the literature to solubilise the protein by means of empirical studies. To screen a range of conditions that may achieve this commercially available product such as FoldIt kit can be used to establish the appropriate conditions.

7.2.4.2 Further study of the regulator gene product

\textit{In vivo} analysis strongly suggests that the \textit{dehR} gene controls both \textit{dehD} and \textit{dehL} as shown in Chapter 6. This confirmed the interpretation of the mutational analysis by Leigh (1981) that a single regulator controls \textit{dehD} and \textit{dehL}. However, it is of interest to confirm by foot-printing or gel shift analysis that the regulator gene product does control both dehalogenase genes.
Figure 7.1. pSC1
Arrows: direction of transcription
Hatched: multicloning region

Figure 7.2. pSC530
Bold line: Multi-cloning region
Arrow: direction of transcription of dehE gene
Hatched: multicloning region
Control of \textit{dehE} by the same regulator gene could not be demonstrated due to presumed lack of a putative promoter sequence in the upstream region of the \textit{dehE} gene in pHC774 (\textit{dehE}^+, \textit{dehR}^+). The easiest method to investigate the control of \textit{dehE} by the regulator gene is to make a construct with the \textit{SalI} site that limits the amount of upstream sequence altered and another \textit{SalI} site introduced upstream of the putative \textit{–24/-12} promoter sequence. Production of dehalogenase by this construct can be tested by monitoring the growth of \textit{E.coli} host carrying the plasmid on D,L2-CP and also by carrying out dehalogenase assays. Alternatively using the solubilised \textit{dehR} gene product, regulation of \textit{dehE} can be investigated by \textit{in vitro} analysis (gel shift experiments).

\textbf{7.2.4.3 Mutational study of \textit{dehR} gene in \textit{Rhizobium sp.}}

A possible way to confirm that the identified regulator gene controls all three dehalogenases is to make a mutant \textit{Rhizobium sp.} lacking \textit{dehR} function and see whether the organism is able to grow on 2,2DCP and D,L2-CP.

The procedure involves deactivation of the proposed \textit{dehR} (e.g. pFH772 or pFH18) by introducing Kanamycin or Tetracycline resistance gene into \textit{dehR}. The disrupted \textit{dehR} plasmid (suicide plasmid) can be used to transform \textit{Rhizobium sp.} by electroporation. In the host organism crossover events that exchange genetic information between the plasmid and the chromosomal DNA would result in a mutated chromosomal \textit{dehR}.

\textbf{7.2.5 \textit{Rhizobium sp.} dehalogenase gene regulation}

As already known the Specificity Constants for D,L2-CP were highest for DehL and DehD. This suggests D,L2-CP is a better substrate for both DehD and DehL than for DehE. Therefore, it is worth investigating the expression of the three dehalogenase genes in cells grown on low and high D,L2-CP concentration. There is a possibility that only DehL and DehD are produced in cells grown on a low concentration because D,L2-CP is a better substrate for DehL and DehD.

pHC773 (\textit{dehR}^+, \textit{dehD}^+, \textit{dehL}^+) in BL21(DE3) clearly showed that the presence of D,L2CP was needed for production of DehD and DehL. It is possible to use this system to check which other compounds can serve as co-inducers. However, the limitation of the system is the ability of the \textit{E.coli} host to take-up the different
halogenated substrates. This up-take problem was shown by the inability of BL21(DE3) pJS771 (dehE+) to grow on 2,2DCP even though the construct could produce DehE (data not presented).

7.2.6 Western blot analysis
The inability of NM522 (pHC2) to produce DehR was inferred from the lack of growth on D,L2CP. Western blotting could be used to confirm the absence of DehR in such conditions. In addition, Western blot analysis might be useful to detect the presence of DehR in cell-free extract from BL21(DE3)[pHC773] (dehR+, dehD+, dehL+) grown in 20mM D,L2-CP.
APPENDICES
Appendix 1: Michaelis-Menten plot

Program used: Microcal Origin version 6.0

Cell-free extract containing: DehL

Substrate: L-2CP

![Michaelis-Menten plot for L-2CP substrate]

Substrate: D,L2-CP

![Michaelis-Menten plot for D,L2-CP substrate]
Substrate: D,L,2,3-DCP

![Graph showing substrate concentration vs. reaction rate](image)

Km = 0.02 ± 0.01

Substrate: DCA

![Graph showing substrate concentration vs. reaction rate](image)

Km = 0.13 ± 0.04
Substrate: DBA

Cell-free extract containing: DehF

Substrate: D-2CP
Substrate: L-2CP

![Graph showing reaction rate vs substrate concentration for L-2CP. The graph includes data points and a fitted line. The Km value is 0.56 ± 0.09.]

Substrate: D,L2-CP

![Graph showing reaction rate vs substrate concentration for D,L2-CP. The graph includes data points and a fitted line. The Km value is 0.41 ± 0.05.]

Substrate: 2,2DCP

Substrate: D,L2,3-DCP
Substrate: MCA

$K_m = 1.88 \pm 0.99$

Substrate: DCA

$K_m = 0.51 \pm 0.11$
Substrate: TCA

Substrate: MBA
Substrate: DBA

\[ \text{Km} = 0.89 \pm 0.21 \]

Substrate: TBA

\[ \text{Km} = 0.22 \pm 0.06 \]
Cell-free extract containing: DehD

Substrate: D-2CP

![Graph for D-2CP substrate](image)

Substrate: D,L-2CP

![Graph for D,L-2CP substrate](image)
Substrate: D,L2,3-DCP

![Graph showing the relationship between substrate concentration and enzyme activity for D,L2,3-DCP with the Michaelis-Menten constant (Km) of 0.53 ± 0.06 mM.]

Substrate: MCA

![Graph showing the relationship between substrate concentration and enzyme activity for MCA with the Michaelis-Menten constant (Km) of 1.02 ± 0.34 mM.]

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Pure enzyme: DehL

Substrate: L-2CP

Substrate: L-2BP
Substrate: D,L2,3-DCP

![Graph of D,L2,3-DCP substrate with Km 0.03±0.01]

Substrate: MCA

![Graph of MCA substrate with Km 0.13±0.01]
Substrate: DBA

Pure enzyme: DehE

Substrate: D-2CP
Substrate: D-2BP

\[
\text{Km} = 0.46 \pm 0.04
\]

Substrate: L-2CP

\[
\text{Km} = 0.41 \pm 0.14
\]
Substrate: L-2BP

![Graph showing substrate L-2BP with Km 0.29 ± 0.05 (unit: mM)].

Substrate: D,L2-CP

![Graph showing substrate D,L2-CP with Km 0.35 ± 0.03 (unit: mM)].
Substrate: D,L-2BP

![Graph showing substrate concentration vs. 
μmol Br/ml/min with Km = 0.22 ± 0.04 mM.]

Substrate: 2,2DCP

![Graph showing substrate concentration vs. 
μmol Cl/ml/min with Km = 0.19 ± 0.03 mM.]

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Substrate: D,L2,3-DCP

Substrate: MCA
Substrate: MBA

Km 2.18 ± 0.30

Substrate: DBA

Km 0.88 ± 0.14
Substrate: TBA

Substrate: D-2CP

Pure enzyme: DehD
Substrate: D-2BP

![Graph showing substrate concentration vs. reaction rate for D-2BP. The x-axis represents mM, and the y-axis represents µmol Br/ml/min. The graph includes a data point at Km 0.48±0.09.](image)

Substrate: D,L2-CP

![Graph showing substrate concentration vs. reaction rate for D,L2-CP. The x-axis represents mM, and the y-axis represents µmol Cl/ml/min. The graph includes a data point at Km 0.04±0.01.](image)
Substrate: D,L2-BP

Km 0.40±0.04

Substrate: D,L2,3-DCP

Km 0.38±0.11

μmol Br/ml/min

Km 0.40±0.04

Substrate: D,L2,3-DCP

Km 0.38±0.11
Substrate: MCA

Substrate: MBA
Appendix II: Complete Nucleotide Sequence for pFH45(dehR) incomplete
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**Restriction Enzymes and Sequences**

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Appendix V: Nucleotide sequence obtained from pSC2(dehD\(^+\), dehL\(^+\))


277


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