Biodegradation (2005) 16: 485–492

Biochemical and molecular characterisation of the 2,3-dichloro-1-propanol dehalogenase and stereospecific haloalkanoic dehalogenases from a versatile *Agrobacterium* sp.

Timothy P. Higgins[1,*], Stephen J. Hope[1], Agus J. Effendi[1,2], Shula Dawson[1] & Brian N. Dancer[1]
[1]*Cardiff School of Biosciences, Cardiff University, Cardiff CF10 3TL, UK;* [2]*Present address: Inter University Center in Biotechnology, Institute Technology Bandung, Bandung 40132, Indonesia (*author for correspondence: e-mail: higgins tp1@cf.ac.uk)*

Accepted 1 November 2004

Key words: Agrobacterium, dehalogenase, dichloropropanol, 2-monochloropropionic acid, *Rhizobium*

Abstract

We previously reported the presence of both haloalcohol and haloalkanoate dehalogenase activity in the *Agrobacterium* sp. strain NHG3. The versatile nature of the organism led us to further characterise the genetic basis of these dehalogenation activities. Cloning and sequencing of the haloalcohol dehalogenase and subsequent analysis suggested that it was part of a highly conserved catabolic gene cluster. Characterisation of the haloalkanoate dehalogenase enzyme revealed the presence of two stereospecific enzymes with a narrow substrate range which acted on D-2-chloropropionic and L-2-chloropropionoic acid, respectively. Cloning and sequencing indicated that the two genes were separated by 87 bp of non-coding DNA and were preceded by a putative transporter gene 66 bp upstream of the D-specific enzyme.

Introduction

Halogenated organic compounds are an extremely important class of environmental chemicals which occur both naturally and increasingly as xenobiotics in the biosphere (Gribble 1996). Many microorganisms have been isolated which utilise these compounds as sole sources of carbon and energy (Fetzner & Lingens 1994; Janssen et al. 1994). Growth on halogenated compounds requires cleavage of the carbon–halogen bond and in some cases this cleavage may be fortuitous (Fetzner & Lingens 1994). However, in many cases specific enzyme mechanisms have evolved to catalyse the cleavage of the carbon–halogen bond (Janssen et al. 1994). The most studied dehalogenation mechanism is the hydrolytic cleavage reaction (Van Hylckama Vlieg et al. 2001). This is particularly true in the case of aliphatic compounds such as haloalkanes, haloalcohols and haloalkanoic acids, where the enzymes involved in the hydrolysis of these compounds have been divided into two sub-groups (Slater et al. 1995).

Previous studies have reported many microorganisms with more than one dehalogenase enzyme activity (Janssen et al. 1994), however, in most cases they are usually specific for the compound used to isolate them. To our knowledge the organism *Xanthobacter autotrophicus* strain GJ10 was the first isolated with dehalogenase activity against two aliphatic compound types, namely haloalkanes and haloalkanoic acids (Janssen et al. 1985). Recently a strain of *Mycobacterium* was isolated which had dehalogenase activity against both haloalkanes and haloalcohols (Poelarends et al. 1999).

We recently isolated a bacterium with the ability to grow on 2,3-dichloro-1-propanol (2,3-DCP) as sole carbon source. It proved to have a wide substrate specificity including dehalogenase activity against both haloalcohols and haloalkanoic acids (Effendi et al. 2000). The organism was identified as a member of the *Rhizobium* family by

16S rRNA homology, was also able to utilise 1,3-dichloro-2-propanol (1,3-DCP) and 2-monochloropropionic acid (2-MCPA) as sole sources of carbon and energy. Other tests indicated that the strain was a strain of *Agrobacterium*. It was found to possess at least four dehalogenase enzymes (Effendi et al. 2000). The 2,3-DCP enzyme was characterised and found to be constitutively expressed whilst all the other dehalogenases were found to be inducible. This paper extends our knowledge on this versatile organism by providing sequence data for the 2,3-DCP enzyme as well as characterising the haloalkanoic dehalogenase enzymes.

Materials and methods

Chemicals

All halogenated compounds were purchased from Sigma (Poole, UK) or Fisher (Loughborough, UK). Unless otherwise stated, other chemicals and media were from Merck (Poole, UK) or Oxoid (Basingstoke, UK). DEAE-Sepharose CL-6B and Sephacryl S-200-HR were purchased from Pharmacia Biotech (UK).

Bacterial strains, plasmids and growth conditions

Cultures of *Agrobacterium* strain NHG3 previously described in Effendi et al. (2000) were routinely grown at 30 °C on SBS medium (Slater et al. 1979). The media was supplemented with the racemic mixture of 2-MCPA or 2,3-DCP as sole source of carbon (filter sterilised to a final concentration of 0.5 g C l^{-1}). *E. coli* XL1-blue (Stratagene) was used as a host for cloning experiments and was maintained at 37 °C in Luria broth (LB) supplemented with 0.5% NaCl. Plasmid pUC18 (Northumbria Biological Ltd, UK) was used as a cloning vector. Recombinant *E. coli* cells were grown at 30 °C in ¼ strength LB (no salt) supplemented with IPTG (0.5 mM) and 2-MCPA (0.5 g Cl^{-1}) and ampicillin (50 μg ml^{-1}).

Dehalogenase activities and protein analysis

Qualitative assays of dehalogenase activities were performed using electrophoretic zymograms, based on colorimetric detection of chloride released by silver nitrate (Weightman & Slater 1980). Quantitative dehalogenase assays of whole cells were detected by measurement of chloride release (Effendi et al. 2000) from 10 ml cultures of ¼ strength LB without added NaCl grown aerobically at 30 °C. Cell-free extracts were prepared by harvesting mid-log phase cultures by centrifugation at 10,000 × *g* for 15 min. The cells were washed and resuspended in 50 mM Tris–H_2SO_4 (pH 8.0) prior to breakage by two passages through a French pressure cell. Cell debris and unbroken cells were removed from the soluble fraction by centrifugation at 45,000 × *g* for 45 min. Dehalogenase activity of partially purified enzyme was measured in a total volume of 5 ml which contained 0.2 M Tris–H_2SO_4 (pH 8.0) and variable amounts of either L- or D-MCPA as substrate (3 replicates for each substrate concentration). The resultant chloride release was measured as described above. Apparent K_m and V_{max} were estimated using the Linweaver–Burke equation.

Protein determination was measured by the biuret method with bovine serum albumin as the standard (Gornall et al. 1949).

One unit of enzyme activity was defined as the amount of enzyme that catalysed the formation of 1 μmol of halide min^{-1}.

Enzyme purification

Unless otherwise stated, all procedures were carried out at 4 °C. Cell-free extracts were fractionated by the stepwise addition of solid $(NH_4)_2SO_4$ from 45–80% of saturation at 0 °C. The precipitate fraction was dialysed overnight against 10 mM Tris–H_2SO_4, 1 mM dithiothreitol (pH 8.0, buffer A). The dialysed fraction was then applied to a DEAE Sepharose CL-6B anion exchange column. The column was washed with buffer A overnight to remove any unbound protein. The bound proteins were then eluted from the column in a total of 500 ml of buffer A with a linear gradient of 0–0.5 M $(NH_4)_2SO_4$. A total of 80 fractions were collected and assayed as described in Effendi et al. (2000). Fractions containing the separated 2-MCPA dehalogenase activities were pooled individually for use in characterisation experiments.

Molecular weight determination

Relative molecular weight determinations under denaturing conditions were performed by SDS-PAGE on a 12% gel (Laemmli 1970) and

under non-denaturing conditions by gel filtration on a Sephacryl S-200-HR column.

DNA manipulation and transformation

All routine DNA manipulation and analysis were carried out as described in Sambrook et al. (1989). Transformation of *E. coli* XL1-Blue supercompetent cells with plasmid DNA was performed as recommended by the supplier.

Cloning strategy for isolation of 2,3-DCP and 2-MCPA genes

Agrobacterium NHG3 genomic DNA was partially digested with *Eco*RI prior to ligation with pUC18 plasmid vector. The ligated DNA was transformed into *E. coli* XL1-blue host cells which were then spread onto LB/ampicillin agar plates with 40 μl IPTG (20 mg ml^{-1}) and 40 μl X-Gal (2% w/v in *N, N′*-dimethylformamide) for selection. Putative clones (white colonies) were then screened for 2,3-DCP dehalogenase and 2-MCPA dehalogenase activity by a colorimetric method (Bergmann & Sanik 1957).

To ensure possible clones were not overlooked by the colorimetric method, transformed *E. coli* XL1-blue cells were grown for 2 days at 30 °C before all the cells were washed-off with 1 ml of SBS medium. Aliquots (150 μl) of the mixed cells were inoculated into ¼ strength LB (no salt)/ampicillin supplemented with IPTG (0.5 mM) and either 2,3-DCP or 2-MCPA (0.5 g carbon l^{-1}). Growth of the cultures at 30 °C was monitored regularly, as was the presence of dechlorinating activity. Dechlorinating cultures were subcultured into the same medium once the cultures had dechlorinated 90% of the theoretical maximum. At each subculture, the cells were plated onto LB/ampicillin plates in order to obtain single colonies, which were then re-inoculated into fresh liquid medium and checked for dechlorinating activity.

Purified plasmid DNA of recombinant cells with dehalogenase activities were digested with a range of restriction enzymes including *Eco*RI, *Bam*HI, *Pst*I and *Hind*III to determine the size of the plasmid DNA.

Nucleotide sequence determination and alignment

An automated DNA sequencer (Applied Biosystems 370A DNA sequencer) was used to determine the DNA sequence of both strands of the putative clone. Initially primers SP6 and T7 were used for sequencing. New oligonucleotide primers were then designed according to the obtained sequence data. DNA sequences were assembled with the aid of ProSeq v2.8 (Filatov et al. 2000). The nucleotide sequence and predicted amino acid sequences were analysed using Expasy Molecular biology server (http://www.expasy.ch/). Nucleotide sequences and predicted amino acid sequences were compared by BLAST (Altschul et al. 1997) searches of the NCBI databases using the default settings of both algorithms.

Nucleotide sequence accession number

The nucleotide sequences presented here have been submitted to the EMBL database under accession numbers AJ488570–AJ488574.

Results

Growth of NHG3 on DL-2-MCPA

Strain NHG3 grew well on the racemic mixture of 2-MCPA with over 80% of theoretical chloride release (Figure 1), indicating that both stereoisomers acted as sources of carbon and energy for growth. Two dehalogenase enzymes were visualised by gel zymography when developed with the DL-2-MCPA (Figure 2), these were initially named 2-MCPA dehalogenase I and II, respectively. Experiments with the individual stereoisomers indicated that strain NHG3 dechlorinated the L-2-MCPA at roughly twice the rate of D-2-MCPA (data not shown), suggesting that enzymes may stereoselectively dechlorinate DL-2-MCPA. This was confirmed by gel zymography where individual isomers were used to show activity (Figure 2).

Enzyme purification and biochemical characterisation

The two 2-MCPA dehalogenases from strain NHG3 were partially purified using two purification steps. The dehalogenase I and II were separated from each other and any haloalcohol dehalogenase activities by anion exchange. The two partially purified dehalogenases were visual-

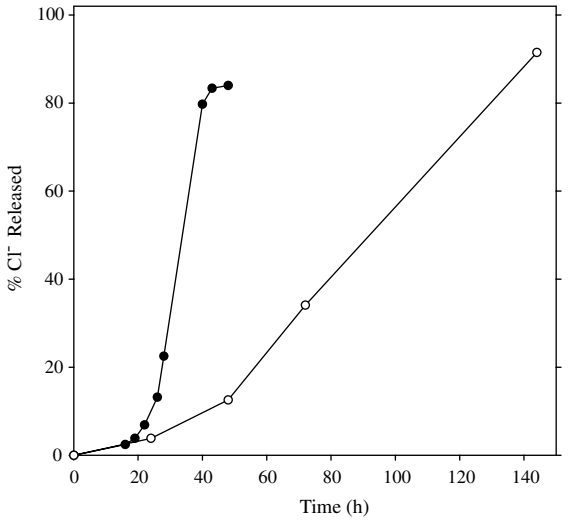

Figure 1. Chloride release from 2-MCPA grown cultures of *Agrobacterium* sp. NHG3 (●) and *E. coli* XL1 blue containing plasmid SJH1 (○).

ised on an activity gel, which clearly showed each enzyme preparation was free from other contaminating dehalogenase activities.

The activity of the partially purified dehalogenases against a range of substrates was tested

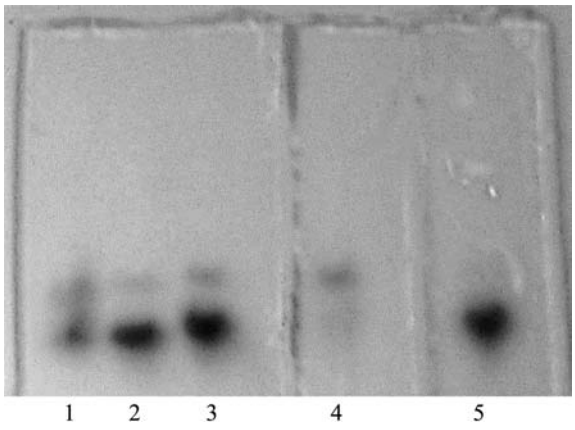

Figure 2. Dehalogenase zymograms of *Agrobacterium* sp. NHG3 and *E. coli* XL1 blue containing plasmid SJH1 cell free extracts grown on 2-MCPA as sole carbon source and developed with racemic 2-MCPA or the D or L isomers of 2-MCPA. Lane 1, *E. coli* XL1 blue containing plasmid SJH1 cell free extract developed with 50 mM DL-2MCPA; Lane 2 and 3, *Agrobacterium* sp. NHG3 cell free extract developed with 50 mM DL-2MCPA; Lane 4, *Agrobacterium* sp. NHG3 cell free extract developed with 50 mM D-2MCPA; Lane 5, *Agrobacterium* sp. NHG3 cell free extract developed with 50 mM L-2MCPA.

Table 1. Enzyme specificity of 2-MCPA dehalogenases. The specific activity of 2-MCPA Deh 1 and Deh 2 against DL-2MCPA, 0.41 U mg^{-1} and 1.42 U mg^{-1} were taken as 100%

Substrate	2-MCPA Deh 1 relative activity (%)	2-MCPA Deh 2 relative activity (%)
DL-2-MCPA	100	100
D-2-MCPA	115	4
L-2-MCPA	7	118
3-MCPA	0	0
2,2-DCPA	2	3
Chloroacetic acid	0	0
1,3-DCP	0	0
2,3-DCP	0	0
3-CPD	0	0

(Table 1). The results showed that both enzymes had narrow substrate specificities and that, as suggested, they were stereospecific with regard to D- and L-2-MCPA. With this in mind we have renamed the dehalogenase I enzyme D-2-MCPA dehalogenase and the dehalogenase II enzyme L-2-MCPA dehalogenase.

Molecular weight determination of D-2-MCPA dehalogenase under native conditions was 53 kDa suggesting that the enzyme was a dimeric protein consisting of two identical subunits of 29 kDa

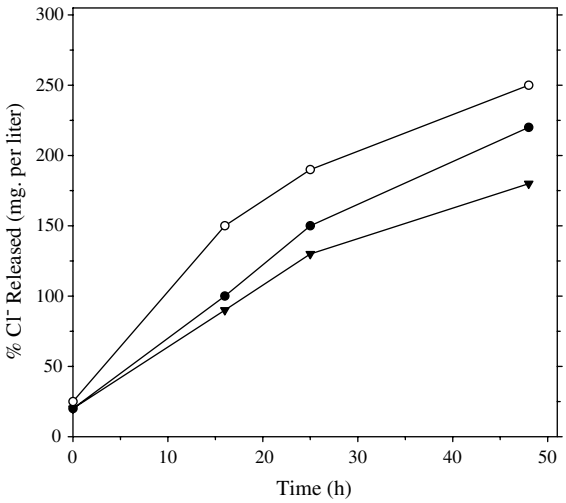

Figure 3. Chloride release from *E. coli* XL1 blue containing plasmid AJE1 cultures grown in SBS medium supplemented with 0.5 g carbon l^{-1} 2,3-DCP (●), 1,3-DCP (○) and CPD (▼).

(denatured molecular weight). The apparent K_m and V_{max} of D-2-MCPA dehalogenase towards D-2-MCPA were 1.9 ± 0.44 mM and 0.49 ± 0.084 U mg^{-1}, respectively.

Molecular weight determination of L-2MCPA dehalogenase under native and denatured conditions indicated that it was a monomeric protein of 31 kDa. The apparent K_m and V_{max} of L-2MCPA dehalogenase towards L-2MCPA were 19.5 ± 0.34 mM and 4.2 ± 0.02 U mg^{-1}, respectively.

Cloning of 2,3-DCP and 2-MCPA dehalogenase genes

Screening of more than 2500 clones generated led to the isolation of a single clone with 2,3-DCP dehalogenase activity (AJE1) (Figure 3). Digestion of the plasmid AJE1 with *Eco*R1 produced two fragments of 2.7 kb (vector), 1.4 kb, respectively. Plasmid AJE1 was then sequenced.

No 2-MCPA-degrading clones were detected by screening using the colorimetric method (Bergmann & Sanik 1957). Therefore to ensure that any slow growing putative 2-MCPA clones were not missed the second screening method was used. Recombinant *E. coli* XL1-blue cells inoculated into ¼ strength LB (no salt)/ampicillin supplemented with IPTG (0.5 mM) and 2-MCPA (0.5 g carbon l^{-1}) were shown to have dechlorinating activity when compared to untransformed *E. coli* XL1-blue cells (Figure 1). Single colonies with the ability to dechlorinate 2-MCPA were isolated. The plasmid DNA isolated from the recombinant clones was shown to contain an insert of approximately 11 kb (data not shown). Digestion of the *Eco*R1 generated clone with *Bam*HI produced two bands on an agarose gel of approximately 6.0 and 7.7 kb, respectively. Re-ligation and transformation of the *Bam*H1 digested plasmid led to the production of numerous recombinants, which were capable of dechlorinating 2-MCPA to over 90% of the theoretical maximum. Analysis of the recombinant cells indicated that they contained a plasmid of approximately 6.0 kb, and therefore and insert of approximately 3.3 kb (vector 2.7 kb). The plasmid designated SJH2 was then sequenced.

Sequence analysis of the 2,3-DCP dehalogenase gene

Analysis of the assembled sequencing data of the AJE2 plasmid revealed that the cloned DNA was 1402 bp in length. An open reading frame (ORF) starting 466 nucleotides from the *Eco*R1 site and was preceded by a putative ribosome binding site (RBS) located between −8 and −12 relative to the start codon (EMBL AJ488570). The ORF consisted of 254 codons encoding a peptide with a predicted molecular mass of 27,951 Da. This corresponds very well with the value of 28.5 kDa estimated by SDS/PAGE (Effendi et al. 2000). A homology search of the NCBI protein database revealed the enzyme designated 2,3-DCP dehalogenase had 100% identity with a 1,3-DCP dehalogenase enzyme from *Agrobacteriun radiobacter* strain AD1 (Van Hylckama Vlieg et al. 2001). Further sequencing in the region upstream of the dehalogenase gene by chromosome walking using restriction site PCR (Weber et al. 1998) confirmed the presence of an epoxide hydrolase enzyme which also had 99% identity with the same enzyme from *A. radiobacter* strain AD1 (Rink et al. 1997; EMBL AJ488571). The 2,3-DCP dehalogenase also had a high degree of identity (91%) to the HalB enzyme of *A. tumefaciens* strain HK7 (Lewis 1996).

Sequence analysis of the 2-MCPA dehalogenase genes

Analysis of the assembled sequencing data of the SJH2 plasmid revealed that the cloned DNA was 3158 bp in length. The sequence data revealed the presence of three coding regions larger than 500 bp, in the same orientation. The first coding region from nucleotide 1 to 514 was incomplete and had a predicted amino acid sequence with a high degree of identity (up to 74%) to the C-terminal region of small molecule transport proteins (EMBL AJ488572) (Goodner et al. 2001). The next coding region began 66 bp downstream of the putative transporter gene and was an open reading frame (ORF1) 257 codons long preceded by a putative RBS between nucleotides −13 and −18 relative to the start codon. ORF1 encoded a protein with a predicted molecular mass of 28,838 Da, which corresponds very well to the value obtained for the D-specific dehalogenase enzyme estimated by SDS/PAGE of 29 kDa (EMBL AJ488573). Homology

searches carried out with the deduced amino acid sequence indicated that it is likely to be a D-specific 2-MCPA dehalogenase enzyme; the two best matches being those of the only two previously described D-specific dehalogenase enzymes (Cairns et al. 1996; Jones et al. 1992). The third coding region began some 87 bp downstream of the putative D-specific dehalogenase gene and was an open reading frame (ORF2) some 275 codons in length (EMBL AJ488574). The start codon of ORF2 was preceded by a putative RBS located between nucleotides −14 and −18 upstream, there was also a putative terminator sequence located downstream of the stop codon of ORF2. The estimated molecular mass of the predicted peptide encoded by ORF2 was 30,906 Da suggesting that this gene expressed the L-specific 2-MCPA dehalogenase enzyme (estimated to be 31 kDa). A comparison of the deduced amino acid sequence with the NCBI protein database revealed that the best matches were with L-specific 2-MCPA dehalogenase enzymes (Cairns et al. 1996; Hill et al. 1999).

Discussion

Agrobacterium strain NHG3 was isolated for it ability to utilise 2,3-DCP as sole source of carbon and energy. Preliminary studies found the organism grew on a wide range of halogenated compounds (Effendi et al. 2000). Microorganisms have previously been isolated which have the ability to utilise haloalkanes and either the resultant haloalcohol or haloacid (Janssen et al. 1985; Poelarends et al. 1999). The presence of the differing dehalogenase enzymes in *Agrobacterium* strain NHG3 is not a consequence of metabolic intermediates, and is more likely to be a common phenomenon which has previously been unreported in the literature.

Agrobacterium strain NHG3 has been shown to possess two haloalcohol dehalogenase enzymes, one of which is an inducible enzyme whilst the other was constitutively expressed (Effendi et al. 2000). The ability to dehalogenate 2,3-DCP was restricted to the constitutively expressed enzyme, which has been biochemically characterised (Effendi et al. 2000). Sequence data revealed that the 2,3-DCP dehalogenase enzyme was identical to the halohydrin dehalogenase enzyme from *A. radiobacter* strain AD1 (Van Hylckama Vlieg et al. 2001) and also had a high degree of identity to the *halB* gene of *A. tumefaciens* (Lewis 1996). The latter two strains were isolated from enrichments on 1,3-DCP, but both were able to dechlorinate 2,3-DCP. While strain NHG3 was isolated for the ability to grow on 2,3-DCP the purified enzyme DCP dehalogenase proved to share with the halohydrin hydrolase of *A. radiobacter* strain AD1 the ability to dehalogenate 1,3-DCP as well as 3-chloro-1,2-propanediol (3-CPD). These enzymes were cloned into *E. coli* on fragments with only a small portion of the nearby epichlorohydrin hydrolase, the recombinant strains were able to dechlorinate both DCP and the resultant 3-CPD (Lewis 1996; Van Hylckama Vlieg et al. 2001; and this study). This would suggest that the epoxide hydrolase activity is not essential for dechlorination to take place even though the dehalogenase enzyme of *Agrobacterium* NHG3 has been shown to degrade both stereoisomers of 2,3-DCP (Effendi et al. 2000). Moreover, the distance between the C-terminus of the epoxide hydrolase and the N-terminus of the haloalcohol dehalogenase, combined with the presence of a possible −24, −12 promoter for the latter gene, would suggest that the two genes are not co-transcribed in an operon but rather form a catabolic cassette. Isolation of geographically distinct microorganisms with highly conserved catabolic gene cluster would suggest that these organisms recently obtained it as a pre-assembled unit from a common ancestral bacterial strain (Poelarends et al. 2000).

Characterisation of the 2-MCPA dehalogenase enzymes clearly showed that each was active against individual stereoisomers of opposite orientation, to our knowledge this ability has only been described in two other microorganisms (Barth et al. 1992; Cairns et al. 1996). Biochemical and molecular characterisation of D-2-MCPA dehalogenase showed it to be a dimeric enzyme as is the case with the majority of 2-haloalkanoate dehalogenase enzymes isolated to date (Slater et al. 1997). Significantly the two best matches by homology were the D-specific dehalogenases DehD (Cairns et al. 1996) and HadD (Jones et al. 1992). A great deal of the amino acid sequence of D-2-MCPA was identical to that of DehD, 59% overall compared to 36% of HadD with large regions showing 100% identity suggesting that D-2-MCPA is closely related to DehD. Subsequent analysis of the nucleotide sequence of the D-2-MCPA enzyme

showed it to share over 90% identity with the coding sequence of *deh*D; such a high degree of sequence identity can only be explained by lateral gene transfer. A recent article (Hill et al. 1999) attempted to classify the haloalkanoate dehalogenase enzymes into gene families. Analysis of the sequence data showed that D-2-MCPA belonged to the group I *deh* genes sharing four of the five essential amino acids identified by Nardi-Dei et al. (1997).

The L-2MCPA is a monomeric enzyme, which is unusual; the only other reported monomeric 2-haloalkanoate dehalogenase enzyme being the L-specific DhlB of *X. autotrophicus* GJ10 (van der Ploeg et al. 1991). A search of the NCBI protein databases revealed that the best match was the DehL enzyme cloned by Cairns et al. (1996) with large regions being perfect matches. The nucleotide sequence showed >90% identity to the coding region of *deh*L. In terms of the evolutionary origins, the L-2-MCPA enzyme does not appear to fall into either of the three gene families outlined by Hill et al. (1999). Since it is closely related to the *deh*L gene of *Rhizobium* sp., we must concur with Hill et al. (1999) that these two genes represent an as yet uncharacterised fourth *deh* family. Closer analysis of the sequencing data would suggest that D-2-MCPA and L-2-MCPA genes are part of an operon with an upstream transport protein and possibly a regulator protein as has been shown with other 2-haloalkanoate dehalogenase genes (Barth et al. 1992; Cairns et al. 1996; Hill et al. 1999). This could be determined by further sequencing upstream of clone SJH2.

In conclusion, *Agrobacterium* sp. NHG3 has two highly conserved dehalogenase gene clusters which bestow a unique characteristic in terms of the range of chlorinated aliphatic compounds it can utilise. Future work will attempt to clarify whether the two operons are located in close proximity on the genome of *Agrobacterium* sp. NHG3 and if so were they obtained together from a common ancestor.

Acknowledgements

This work was supported financially by the British Council and The European Regional Development Fund.

References

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W & Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25: 3389–3402

Barth PT, Bolton L & Thompson JC (1992) Cloning and partial sequencing of an operon encoding two *Pseudomonas putida* haloalkanoate dehalogenases of opposite stereospecificity. J. Bacteriol. 174: 2612–2619

Bergmann JG & Sanik J (1957) Determination of trace amounts of chloride in naphtha. Anal. Chem. 29: 241–243

Cairns SS, Cornish A & Cooper RA (1996) Cloning, sequencing and expression in *Escherichia coli* of two *Rhizobium* sp. Genes encoding haloalkanoate dehalogenases of opposite stereospecificity. Eur. J. Biochem. 235: 744–749

Effendi AJ, Greenaway SD & Dancer BN (2000) Isolation and characterisation of 2,3-dichloro-1-propanol-degrading rhizobia. Appl. Environ. Microbiol. 66: 2882–2887

Fetzner S & Lingens F (1994) Bacterial dehalogenases: biochemistry, genetics, and biotechnological applications. Microbiol. Rev. 58: 641–685

Filatov DA, Moneger F, Negrutiu L & Charlesworth D (2000) Low variability in a Y-linked plant gene and its implications for Y-chromosome evolution. Nature 404: 388–390

Goodner B, Hinkle G, Gattung S, Miller N, Blanchard M, Qurollo B, Goldman BS, Cao Y, Askenazi M, Halling C, Mullin L, Houmiel K, Gordon J, Vaudin M, Iartchouk O, Epp A, Liu F, Wollam C, Allinger M, Doughty D, Scott C, Lappas C, Markelz B, Flanagan C, Cromwell C, Gurson J, Lomo C, Sear C, Strub G, Cielo C & Slater S (2001) Genome sequence of the plant pathogen and biotechnology agent *Agrobacterium tumefaciens* C58. Science 294: 2323–2328

Gornall AG, Bardawill CJ & David MM (1949) Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177: 751–766

Gribble GW (1996) The diversity of natural organochlorines in living organisms. Pure Appl. Chem. 68: 1699–1712

Hill KE, Marchesi JR & Weightman AJ (1999) Investigation of two evolutionary unrelated halocarboxylic acid dehalogenase gene families. J. Bacteriol. 181: 2535–2547

Janssen DB, Scheper A, Dijkhuizen L & Witholt B (1985) Degradation of halogenated aliphatic compounds by *Xanthobacter autotrophicus* GJ10. Appl. Environ. Microbiol. 49: 673–677

Janssen DB, Pries F, vander Ploeg J, Kazemier B, Terpstra P & Witholt B (1994) Genetics and biochemistry of dehalogenating enzymes. Ann. Rev. Microbiol. 48: 163–191

Jones DHA, Barth PT, Byrom D & Thomas CM (1992) Nucleotide sequence of the structural gene encoding a 2-haloalkanoic acid dehalogenase of *Pseudomonas putida* strain AJ1 and purification of the encoded protein. J. Gen. Microbiol. 138: 675–683

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

Lewis MR (1996) Cloning and nucleotide sequence of the 1,3-dichloro-2-propanol and 3-chloro-1,2-propanediol dehalogenase gene of *Agrobacterium tumefaciens* HK7. MPhil Thesis, University of Wales, Cardiff, UK

Nardi-Dei V, Kurihara T, Park C, Esaki N & Soda K (1997) Bacterial DL-2-Haloacid dehalogenase from *Pseudomonas* sp. Strain 113: gene cloning and structural comparison with D and L-2 haloacid dehalogenases. J. Bacteriol. 179: 4232–4238

Poelarends GJ, Van Hylckama Vlieg JET, Marchesi JR, Freitas Dos Santos LM, and Janssen DB (1999) Degradation of 1,2-dibromoethane by *Mycobacterium* sp. Strain GP1. J. Bacteriol. 181: 2050–2058

Poelarends GJ, Zandstra M, Bosma T, Kulakov LA, Larkin MJ, Marchesi JR, Weightman AJ & Janssen DB (2000) Haloalkane-utilising *Rhodococcus* strains isolated from geographically distinct locations possess a highly conserved gene cluster encoding haloalkane catabolism. J. Bacteriol. 182: 2725–2731

Rink R, Fennema M, Smids M, Dehmel U & Janssen DB (1997) Primary structure and catalytic mechanism of epoxide hydrolase from *Agrobacterium radiobacter* AD1. J. Biol. Chem. 272: 14650–14657

Sambrook J, Fritsch EF & Maniatis T (1989) Molecular Cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Slater JH, Bull AT & Hardman DJ (1995) Microbial dehalogenation. Biodegradation 6: 181–189

Slater JH, Bull AT & Hardman DJ (1997) Microbial dehalogenation of halogenated alkanoic acids, alcohols and alkanes. Adv. Microb. Physiol. 38: 133–175

Slater JH, Lovatt D, Weightman AJ, Senior E & Bull AT (1979) The growth of *Pseudomonas putida* on chlorinated aliphatic acids and its dehalogenase activity. J. Gen. Microbiol. 114: 125–136

van der Ploeg J, van Hall G & Janssen DB (1991) Characterisation of the haloacid dehalogenase from *Xanthobacter autotrophicus* GJ10 and sequencing of the *dhlB* gene. J. Bacteriol. 173: 7925–7933.

Van Hylckama Vlieg JET, Tang L, Lutje Spelberg JH, Smilda T, Poelarends GJ, Bosma T, van Merode AEJ, Fraaije MW & Janssen DB (2001) Halohydrin dehalogenases are structurally and mechanistically related to short chain dehydrogenases/reductases. J. Bacteriol. 183: 5058–5066

Weber KL, Bolander ME & Sarker G (1998) Rapid acquisition of unknown DNA sequence adjacent to a known segment by multiplex restriction site PCR. BioTechniques 25: 415–419

Weightman AJ & Slater JH (1980) Selection of *Pseudomonas putida* strains with elevated dehalogenase activities by continuous culture growth on chlorinated alkanoic acids. J. Gen. Microbiol. 121: 187–193