The plasmid-located haloalkane dehalogenase gene from *Rhodococcus rhodochrous* NCIMB 13064

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The haloalkane dehalogenase (dhaA) gene from *Rhodococcus rhodochrous* NCIMB 13064 was cloned and sequenced. Its comparison with the previously studied dhlA gene from *Xanthobacter autotrophicus* GJ10 did not show homology. However, the amino acid sequences of the products of these genes showed approximately 30% identity and several of the catalytic amino acid residues were conserved in the NCIMB 13064 dehalogenase. A high level of dhaA expression was demonstrated in *Escherichia coli* cells and this gene was shown to encode a dehalogenase with the activity against chloroalkanes of chain length C_3-C_40. Also, some dehalogenase activity against 1,2-dichloroethane encoded by the cloned dhaA gene was detected. The analysis of NCIMB 13064 derivatives lacking dehalogenase activity showed that the dhaA gene was located on the 100 kbp pRTL1 plasmid. It was also found that reversible rearrangements of DNA in the dhaA region may be responsible for the control of expression of haloalkane dehalogenase in *R. rhodochrous* NCIMB 13064. A number of repeated and inverted sequences which may cause genetic instability at the locus were found in the haloalkane dehalogenase gene region.

**Keywords**: *Rhodococcus rhodochrous*, dhaA, Dha- derivatives, genome rearrangements

**INTRODUCTION**

Many haloalkanes persist in the environment to become major pollutants of the biosphere. Haloalkane dehalogenases have been found in Gram-negative (Keuning et al., 1985) and Gram-positive haloalkane-utilizing bacteria (Keuning et al., 1985; Yokota et al., 1987; Scholtz et al., 1987; Sallis et al., 1990). Haloalkane dehalogenases are unique enzymes which catalyse hydrolytic dehalogenation of corresponding hydrocarbons without participation of oxygen or coenzymes. The haloalkane dehalogenase (DhlA) from *Xanthobacter autotrophicus* GJ10 has been extensively studied and the sequence of the dhlA gene determined (Janssen et al., 1988, 1989). However, very little is known about the genetic organization of haloalkane degradation in Gram-positive bacteria.

*Rhodococcus rhodochrous* NCIMB 13064 can utilize a wide range of 1-haloalkanes as sole carbon and energy source. Short-chain 1-chloroalkanes (C_3-C_8) are metabolized by the initial action of a hydrolytic dehalogenase to produce the corresponding alcohol. However, growth on long-chain 1-chloroalkanes (C_12-C_18) results only in a low level of dehalogenase activity. The attack on the long-chain 1-chloroalkanes is initiated by oxygenase action at the non-halogenated end to produce ω-chlorofatty acids. These are then degraded by β-oxidation (Curragh et al., 1994). Two plasmids, pRTL1 (100 kbp) and pRTL2 (80 kbp), have been found in strain NCIMB 13064; pRTL1 was shown to carry at least some genes for the dehalogenation of short-chain 1-chloroalkanes. No association was found between the utilization of 1-chloroalkanes with chain lengths of C_12-C_18 and the presence of pRTL1 in bacterial cells. This suggested that different genetic determinants are involved in the dehalogenation and utilization of short- and long-chain 1-chloroalkanes (Kulakova et al., 1995). Three separate genetic events independently led to the inability of NCIMB 13064 to dehalogenate short-chain 1-chloroalkanes: either the complete loss of pRTL1; or the integration of pRTL1 into the chromosome; or deletion of a 20 kbp fragment from pRTL1. High-

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frequency transfer of the 1-chloroalkane degradation marker associated with pRTL1 was demonstrated in matings between different derivatives of R. rhodochrous NCIMB 13064 (Kulakova et al., 1995).

The haloalkane dehalogenase from R. rhodochrous NCIMB 13064 has been purified to homogeneity. It is monomeric and its molecular mass was determined to be 33 kDa (Stafford, 1993). An analysis of the physico-chemical characteristics of NCIMB 13064 dehalogenase suggested similarities with other haloalkane halidohydrolases. The first 17 residues of the N-terminal amino acid sequence were identical to those reported for halidohydrolases with a similar substrate range isolated from Rhodococcus erythropolis (Sallis et al., 1990) and Arthrobacter sp. (Scholtz et al., 1987). However, no similarities were detected with the N-terminal amino acid sequence of the DhlA halidohydrolase isolated from Xanthobacter autotrophicus GJ10 (Keuning et al., 1985), which is the most extensively studied dehalogenase to date.

In this paper we report the cloning of a haloalkane dehalogenase gene from Rhodococcus rhodochrous NCIMB 13064, its sequencing and its expression in Escherichia coli. Analysis of some of NCIMB 13064 derivatives unable to utilize chloroalkanes is also presented.

METHODS

Bacterial strains and plasmids. E. coli DH5α (Sambrook et al., 1989), cosmid vector pLAFR5 and plasmid vector pUC129 (Keen et al., 1988) were used for the cloning experiments. R. rhodochrous NCIMB 13064 (sub-strain S14A3), which harbours two plasmids, pRTL1 and pRTL2, was employed in this study. Its derivatives unable to utilize short-chain chloroalkanes, S2 (pRTL2) and P200 (pRTL1 Δ20 kbp, pRTL2), were described previously (Kulakova et al., 1995).

Media and growth conditions. Both R. rhodochrous and E. coli strains were grown in a rich (2YT) or minimal (M9) medium (Miller, 1972). When required, Difco agar (1:8 %, w/v) was added to the medium. Ampicillin (100 μg ml⁻¹), tetracycline (15 μg ml⁻¹), IPTG (50 μg ml⁻¹) and X-Gal (50 μg ml⁻¹) were used for the detection of recombinant plasmids. Rich medium (2YT) without sodium chloride was used to screen for E. coli clones with chloroalkane dehalogenase activity. For the isolation of plasmid and total DNA from R. rhodochrous, strains were grown in YE medium (Kulakova et al., 1995).

DNA techniques. Plasmid DNA from R. rhodochrous strains was isolated as described by Schreiner et al. (1991), and total DNA as described by Kulakova et al. (1995). Recombinant DNA work was done using standard protocols as described by Sambrook et al. (1989). Restriction of both plasmid and total DNA was performed using enzymes obtained from Pharmacia for 12-20 h, according to the manufacturer's instructions.

Agarose gel electrophoresis was carried out using 0.9-1 % (w/v) agarose in TAE buffer at 5-7 V cm⁻¹. Low-melting-point agarose (Bio-Rad) was used for the recovery of DNA fragments. Fragments were then purified by phenol and phenol/chloroform extraction with subsequent ethanol precipitation.

DNA fragments were transferred to Z-probe membranes (Bio-Rad) by Southern blotting, as described by Sambrook et al. (1989). The membranes were then treated according to the manufacturer's instructions. Hybridization probes were labelled with [32P]dCTP using an oligolabelling kit (Pharmacia) and hybridization was carried out in 0.25 M Na₂HPO₄, pH 7.2, 7 % (w/v) SDS at the temperatures indicated in Results for between 6 and 12 h.

Cloning of the dhaA gene from R. rhodochrous. Plasmid DNA (i.e. pRTL1 and pRTL2) isolated from R. rhodochrous NCIMB 13064 was partially digested with restriction endonuclease Sau3A. Fragments of 15-25 kbp were isolated and ligated with cosmid vector pLAFR5 previously digested with BamHI and ScaI. The resultant preparations were packaged into phage λ heads using an in vitro packaging kit (Amersham) and transduced into E. coli DH5α cells. Recombinant clones were tested for dehalogenase activity by release of chloride ions after growth to late exponential phase (at 30 °C; 10 ml cultures) in chloride-free 2YT medium in the presence of 1-chlorobutane (10 mM). Chloride ion release was measured using a Corning Chloride Analyser 926. Sub-cloning of dhaA from Dha⁺ cosmid clones was done using standard methods with plasmid vector pUC129 (Keen et al., 1988).

Measurement of dehalogenase activity. This was assayed in cell-free extracts of R. rhodochrous NCIMB 13064 after growth in minimal medium with 1-chlorobutane (10 mM) as well as E. coli strains grown in 2YT medium. At the end of the exponential phase, cells were harvested, washed and resuspended in 50 mM Tris/H₂SO₄ (pH 8.0). Cultures were disrupted by sonication at 0 °C for 5 min (E. coli) and 12 min (R. rhodochrous) using an MSE Soniprep 150 set at maximum power (peak-to-peak amplitude 12 μm). Preparations were cleared by centrifugation at 35000 g for 1 h at 4 °C. Assay substrate was added to a concentration of 10 mM and chloride ion release was measured using a Corning Chloride Analyser 926. Corrections were made for abiotic chloride release. Protein concentrations were determined by the method of Bradford (1976), using bovine serum albumin as standard.

DNA sequencing. Recombinant pUC129 plasmids with insertions containing the dhaA gene were used as templates for the Taq Dye-Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems). DNA sequences were read on an automatic DNA sequencer (Applied Biosystems, model 373A). The DNA sequence of the dehalogenase region (plasmid pUTL11X) was determined using sub-clones of this plasmid and additional primers. Primer synthesis was performed by Dr C. Stevenson (School of Biology and Biochemistry, The Queen's University of Belfast). The nucleotide sequences of both strands were determined. Computer analysis of the sequences was performed using the DNAsis (Hitachi) software package. Alignment of the protein sequences and analysis of the phylogenetic relationship between corresponding enzymes were performed using the Higgins-Sharp algorithm (Higgins & Sharp, 1988). Searches for nucleotide and amino acid sequence similarities were done by using the FASTA program (Pearson & Lipman, 1988) in the EMBL database.

RESULTS

Cloning of haloalkane dehalogenase gene dhaA

A gene bank of R. rhodochrous NCIMB 13064 plasmid DNA was constructed as described in Methods. Plasmid DNA preparations were used, because our previous results suggested that dehalogenation of short-chain chloroalkanes was associated with one of the two plasmids present in this strain (Kulakova et al., 1995).
Table 1. Dehalogenation of chloroalkanes by R. rhodochrous NCIMB 13064 and E. coli recombinant strains

<table>
<thead>
<tr>
<th>Organism (plasmid)</th>
<th>Specific activity (nmol min⁻¹ mg⁻¹)*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>CIC₃</td>
</tr>
<tr>
<td>R. rhodochrous NCIMB 13064(pRTL1, pRTL2)</td>
<td>40</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
</tr>
<tr>
<td>DH5α(pLTL1k)</td>
<td>7</td>
</tr>
<tr>
<td>DH5α(pLTL1l)</td>
<td>ND</td>
</tr>
<tr>
<td>DH5α(pLTL1lX)</td>
<td>38</td>
</tr>
<tr>
<td>DH5α(pLTL1l12)</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

* Dehalogenation was measured as chloride ion released when assay substrate was added to a concentration of 10 mM to cell-free extracts. CIC₃, 1-chloropropane; CIC₄, 1-chlorobutane; CIC₅, 1-chlorohexane; CIC₆, 1-chlorooctane; CIC₇, 1-chlorononane; CIC₈, 1-chlorodecane; CIC₉, 1-chlorohexadecane; DCE, 1,2-dichloroethane. The results are the means of two experiments. ND, Not determined.

Fig. 1. Restriction map of the dhaA haloalkane dehalogenase gene region. A 5.8 kbp BamHI fragment was sub-cloned into pUC129 (plasmid pUTL11). E. coli cells expressed dehalogenase activity when transformed with this plasmid. Sub-clones of pUTL11 were produced and analysed. RI, EcoRI; RV, EcoRV; K, KpnI; Sm, Smal; X, XbaI; B, BamHI; dhaA, haloalkane dehalogenase gene locus, with direction of transcription (arrow).

Expression of haloalkane dehalogenase DhaA in E. coli

The dehalogenase activity and substrate range were measured for the key clones using resting E. coli cells and cell-free extracts incubated with the appropriate substrate (Table 1). All of the Dha⁺ E. coli clones expressed dehalogenase activity against short-chain (C₃-C₆) chloroalkanes, as well as 1-chlorononane and 1-chlorodecane. Dehalogenase activities in the pUC129-based clones were significantly higher (probably due to a copy number effect) than in the pLAFR5-based ones. In fact, they were even higher than the activities expressed by the original R. rhodochrous strain (Table 1). The effective expression of haloalkane dehalogenase in E. coli was previously shown for the gene cloned from X. autotrophicus GJ10 (Janssen et al., 1989), but in the case of Rhodococcus it was less expected because of the evolutionary distance between these species. Surprisingly, a low level of dehalogenase activity against 1,2-dichloroethane was detected in clones containing...
The nucleotide sequence of the 2118 bp fragment in plasmid pUTL11X encoding the haloalkane dehalogenase gene was determined (Fig. 2). Only one ORF, which could encode a protein of approximately 30 kDa, was found. Correspondence of this ORF to the dhaA gene was confirmed on the basis of the known N-terminal sequence (first 19 amino acids) of the NCIMB 13064 haloalkane dehalogenase (Stafford, 1993). The ORF (nt 752-1633) is preceded by a possible ribosome-binding site (Shine & Dalgarno, 1974) and it encodes a protein of 292 amino acids with a calculated molecular mass of 33,244 Da, corresponding closely with that determined for the purified haloalkane dehalogenase (Stafford, 1993).

Computer analysis of the dhaA region revealed the presence of direct (DR) and inverted (IR) repeats (Fig. 2). The most interesting structural feature was found in the 150 bp region upstream of the dhaA gene, where two overlapping IRs of 16 and 13 bp are present. The IR gene (nt 617–730) in its turn has a complex structure in that 8 bp inverted sequences (AGATGCCG) are present within it. Also the upstream part of the bigger repeat (nt 617) partially overlaps with, and the downstream part (nt 715) completely overlaps with, the DR sequence AGATGCCGCTCTC (nt 617 and 715, Fig. 2). These IRs may form a stable stem-loop structures [the free energy of the nt 617–730 region, calculated using RNA energy values, was \( \Delta G = -33.3 \text{kcal mol}^{-1} \) (139-3 kJ mol\(^{-1}\)) and that for the nt 611–638 repeat was \( \Delta G = -26.4 \text{kcal mol}^{-1} \) (110-5 kJ mol\(^{-1}\)).

A sequence with a structure typical of rho-independent terminators (Carafa et al., 1990) with a predicted free energy (\( \Delta G \)) of -26.2 kcal mol\(^{-1}\) (109.6 kJ mol\(^{-1}\)) was identified 26 nucleotides downstream from the termination codon (Fig. 2).

The nucleotide sequence of the dhaA gene has only 59% similarity in a 301 bp overlap with the dhlA gene from X. autotrophicus GJ10 (Janssen et al., 1989). The deduced amino acid sequence of the dhaA gene exhibited approximately 30% identity with the dhlA product (Fig. 3). Significant similarity was also found with the amino acid sequence of haloacetate dehalogenase (DehH1) from Moraxella sp. (Kawasaki et al., 1992) (Fig. 3).

Analysis of DhaA mutant strains

It was previously shown that short-chain haloalkane degradation in R. rhodochrous NCIMB 13064 was likely to be controlled by the pRTL1 plasmid. However, we had no proof that the dhaA gene was located on this plasmid. In order to locate the haloalkane dehalogenase, a 2 kb XbaI–BamHI fragment of plasmid pUTL11X, which included the dhaA gene with adjacent regions, was purified and used as a probe in Southern hybridization experiments with plasmid and total DNA isolated from NCIMB 13064. Under conditions of high stringency (hybridization at 65 °C for 6 h) hybridization signals were detected with plasmid and total DNA preparations from the parental NCIMB 13064 strain. However, there was no hybridization with DNA from the DhaA S2 derivative, which lacks pRTL1. These results clearly indicate that the haloalkane dehalogenase gene is located on the pRTL1 plasmid.
Previously we reported that the P200 Dha<sup>−</sup> derivative of NCIMB 13064, which had lost a 20 kbp fragment of pRTL<sub>1</sub>, could revert to the original Dha<sup>+</sup> phenotype (revertants were detected in cell populations at a frequency of approximately 10<sup>−7</sup>) with the restoration of pRTL<sub>1</sub> (Kulakova et al., 1995). However, no hybridization of DNA from this strain was detected with the dhaA probe under conditions of high stringency. In this study another spontaneous Dha<sup>−</sup> mutant (P400) was isolated which had lost both pRTL<sub>1</sub> and pRTL<sub>2</sub>. Surprisingly, this strain was also able to revert to the Dha<sup>+</sup> phenotype at a frequency of 10<sup>−5</sup>–10<sup>−6</sup>, with restoration of the original plasmids. It was shown that R. erythropolis Y2 (Sallis et al., 1990) might be similar to that described in this paper according to the N-terminal amino acid sequence similarities of the catalytic residues of DHLA<sub>r</sub> and of haloacetate dehalogenase from Arthrobacter sp. (DEHH<sub>1</sub>). Catalytic residues of DHLA<sub>r</sub> (Asp124, Trp125, Trp260 and His289) are identical in two of the proteins by dots. The sequence representing the cap domain in DHLA<sub>r</sub> is underlined. Residues identical in all three proteins are indicated by asterisks and those identical in two of the proteins by dots.

**DISCUSSION**

There are a number of reports describing microorganisms with the ability to utilize chlorinated hydrocarbons. The haloalkane dehalogenase determinants in Arthrobacter sp. HA1 (Scholtz et al., 1987) and Rhodococcus erythropolis Y2 (Sallis et al., 1990) might be similar to that described in this paper according to the N-terminal amino acid sequence similarities of the enzymes. It was shown that R. erythropolis Y2 harboured a plasmid, although it was not shown to be associated with dehalogenase activity (Sallis et al., 1990). The only haloalkane dehalogenase gene (dhlA) which has been cloned and sequenced to date is from X. autotrophicus GJ10 (Janssen et al., 1989). This gene was found to be located on the plasmid pXAU1 (Tardif et al., 1991). The X-ray structure of the GJ10 haloalkane...
dehalogenase has been determined (Franken et al., 1991; Verschuuren et al., 1993). The enzyme has two domains, and the catalytic residues are situated in the cavity between them, which is reported to be the substrate-binding site (Franken et al., 1991).

The nucleotide sequences of the dhlA and dhaA genes do not exhibit significant homology. However, comparison of the amino acid sequences of the corresponding enzymes and of Moraxella sp. haloacetate dehalogenase (Kawasaki et al., 1992) revealed some important features (Fig. 3). A number of amino acid residues which were shown to play a catalytic role in DhlA are conserved in the NCIMB 13064 dehalogenase. These are: Asp124, which was suggested to be a nucleophilic residue (Pries et al., 1994); and Trp125 (Verschuuren et al., 1993; Pries, 1995) and His289, which were shown to be essential for the hydrolysis of the alkyl–enzyme intermediate (Pries et al., 1995). According to the DNA sequence analysis of dhaA presented here, catalytic Trp175 and Asp260 (charge relay) (Verschuuren et al., 1993; Pries, 1995) were not conserved in the R. rhodochrous NCIMB 13064 haloalkane dehalogenase. Asp124 and His289 residues were also conserved in the DhlH1 haloacetate dehalogenase (Kawasaki et al., 1992). In the region which corresponds to the Cap domain of the DhlA dehalogenase, the similarities are more pronounced in the C-terminal part. The results of the computer analysis of the all three dehalogenases indicated closer evolutionary relations between the two haloalkane dehalogenases than with the haloacetate dehalogenase (matching percentages are 8.5% and 4.4%, respectively).

Other investigators have demonstrated that dehalogenase genes may be located on plasmids (Tardif et al., 1991) or on transposable elements (Slater et al., 1985; Thomas et al., 1992a, b). In this report we demonstrate that the dhaA gene of R. rhodochrous NCIMB 13064 is located on the 100 kbp plasmid pRTL1. This plasmid was previously shown to be transmissible in matings between R. rhodochrous strains (Kulakova et al., 1995). Only dehalogenation of haloalkanes with shorter chains (C3-C10) is encoded by the dhaA gene and the pRTL1 plasmid. A different dehalogenation system(s) is probably involved in the utilization of haloalkanes with longer chains by NCIMB 13064.

In this work we investigated mutations associated with the dhaA locus in two revertible Dha- derivatives. Unexpectedly, we found that in Dha- strains P200 and P400 hybridization of dhaA probe evidently occurred with several regions of the genome. These results suggest that genomic rearrangements in NCIMB 13064 may occur in the region of the haloalkane dehalogenase locus. It is worth noting that genomic rearrangements are well documented in the genus Streptomyces, which represents an extensively studied group of Gram-positive micro-organisms. Genetic instability of these micro-organisms is usually associated with rearrangements of their genomes (Birch et al., 1989; Flett & Cullum, 1987; Schneider et al., 1993). It is also well established that perfect and imperfect direct repeats (McCorkle & Altman, 1982; Lopez et al., 1984) as well as palindromic sequences (DasGupta et al., 1987; Peeters et al., 1988) can increase the frequency of genomic rearrangements in different micro-organisms. Inverted and direct repeats present in the dhaA gene region may be responsible for rearrangements in haloalkane dehalogenase. However, the role of genomic rearrangements in the dhaA region of R. rhodochrous remains to be elucidated.

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Analysis of haloalkane dehalogenase gene


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