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

Anna N. Kulakova, Michael J. Larkin and Leonid A. Kulakov

Author for correspondence: Leonid A. Kulakov (Questor Centre). Tel: +44 1232 335577. Fax: +44 1232 661462. e-mail: l.kulakov@queens-belfast.ac.uk

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 C₃-C₈. 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 (C₃-C₈) are metabolized by the initial action of a hydrolytic dehalogenase to produce the corresponding alcohol. However, growth on long-chain 1-chloroalkanes (C₁₂-C₁₈) 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 C₁₂-C₁₈ 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-
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 physicochemical 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 S1A3), 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 fragments. Fragments were then purified by phenol and phenol/chloroform extraction with subsequent ethanol precipitation.

**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 by 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.

**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>
</thead>
<tbody>
<tr>
<td></td>
<td>ClC₃</td>
</tr>
<tr>
<td><em>R. rhodochrous</em> NCIMB 13064(pRTL1, pRTL2)</td>
<td>40</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
</tr>
<tr>
<td>DHS₅(pLTL1k)</td>
<td>7</td>
</tr>
<tr>
<td>DHS₅(pUTL11)</td>
<td>ND</td>
</tr>
<tr>
<td>DHS₅(pUTL11X)</td>
<td>38</td>
</tr>
<tr>
<td>DHS₅(pUTL112)</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. ClC₃, 1-chloropropane; ClC₄, 1-chlorobutane; ClC₅, 1-chlorohexane; ClC₆, 1-chlorooctane; ClC₇, 1-chlorononane; ClC₈, 1-chlorodecane; ClC₉, 1-chlorohexadecane; DCE, 1,2-dichloroethane. The results are the means of two experiments. ND, Not determined.

### 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-C₈) 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...
A. N. KULAKOVA and OTHERS

**Fig. 2.** Nucleotide sequence of the haloalkane dehalogenase (dhaA) gene region. The nucleotide sequence was obtained from pUTL11X (Fig. 1). The nucleotide sequence was designated by underlining; a rho-independent terminator (nt 660–687) and palindrome sequence in the region upstream of dhaA (nt 611–638) are shown by underlining with arrows.

Nucleotide sequence analysis of the haloalkane dehalogenase gene region

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 33244 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 DR sequence AGATGACCGGTCATCT (nt 617–730) in its turn has overlapping IRs of 16 and 13 bp are present. The IR structure is shown in 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 $\Delta G = -33.3$ kcal mol$^{-1}$ (139-3 kJ mol$^{-1}$) and that for the nt 611–638 repeat was $\Delta G = -26.4$ 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 (AG) 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).

Analysis of Dha$^-$ 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 Dha$^-$ 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- derivative of NCIMB 13064, which had lost a 20 kb fragment of pRTL1, could revert to the original Dha+ phenotype (revertants were detected in cell populations at a frequency of approximately $10^{-5}$) with the restoration of pRTL1 (Kulakova et al., 1995). However, no hybridization of DNA from this strain was detected when the XbaI-BamHI fragment of pUTL11X was used as a probe (data not shown). However, when hybridization was carried out at temperatures above 60°C no signal was detected with strains P200 and P400, and at temperatures below 45°C non-specific hybridization was observed. Hybridization of the same NCIMB 13064, P200 and P400 strains with a 1.5 kbp DNA fragment cloned from a different region of the NCIMB 13064 genome resulted in signals of approximately equal intensity with all DNAs at 50°C and 60°C (results not shown). The results obtained indicated that rearrangements of genetic material may have occurred in the NCIMB 13064 strain in the region of the dhaA locus, and these were probably responsible for the loss of expression of this gene.

### 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

<table>
<thead>
<tr>
<th>DHA</th>
<th>MSEITG</th>
<th>FFDPHYVEVL</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEH</td>
<td>M--------</td>
<td>DFP------</td>
<td>50</td>
</tr>
<tr>
<td>DHLA</td>
<td>MINARYFPDFQSNLDQYPFGNYLFFGNYLGNY</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>DHA</td>
<td>F-LGQTPSYLWNRIHPHA-PKAIIPADFLPGDSKDFP ALIGN</td>
<td>DLDY</td>
<td>100</td>
</tr>
<tr>
<td>DEH</td>
<td>M-LWWQFRAMARVAPQALAEH-HTVCADLRGDSOKPCILDDSN</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>DHLA</td>
<td>LQCCPTEWYSLYRMIPVFASGVRVADPFQGFKDPDFR</td>
<td>VEDSIT</td>
<td>100</td>
</tr>
</tbody>
</table>

![Fig. 3. Amino acid sequence alignment of haloalkane dehalogenases from *R. rhodochrous* NCIMB 13064 (DHA) and *X. autotrophicus* GJ10 (DHLA), and the haloacetate dehalogenase from *Moraxella* sp. (DEHH1). Catalytic residues of DHA (Asp124, Thr125, Thr175, Asp280 and His289) are given in bold letters. The sequence representing the cap domain in DHLA is underlined. Residues identical in all three proteins are indicated by asterisks and those identical in two of the proteins by dots.](image-url)
Fig. 4. Hybridization analysis of the dhaA gene region in R. rhodochrous NCIMB 13064 and its Dha- derivatives. P200 (which has a 20 kbp deletion in plasmid pRTL1: Kulakova et al., 1995) and P400 (which has lost plasmids pRTL1 and pRTL2). Total DNA from NCIMB 13064 (lane 1), P200 (lane 2) and P400 (lane 3) was restricted with BamHI, separated in 0.9% agarose gel and transferred onto Z-probe membrane. Hybridization was performed with the 32P-labeled EcoRI-BamHI fragment isolated from pUTL1X (nt 1178-2118; Fig. 2) at 50 °C for 6 h.

dehalogenase has been determined (Franken et al., 1991; Verschueren 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. halococetic 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 DhIA are conserved in the NCIMB 13064 dehalogenase. These are: Asp124, which was suggested to be a nucleolitic residue (Pries et al., 1994); and Trp125 (Verschueren 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) (Verschueren 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 halococetic dehalogenase (Kawasaki et al., 1992). In the region which corresponds to the Cap domain of the DhIA 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.

ACKNOWLEDGEMENTS

This project was supported by The Queen’s University Environmental Science and Technology Research Centre. We are especially grateful to Dr Clark Stevenson for the synthesis of sequencing primers.

REFERENCES


Analysis of haloalkane dehalogenase gene


Received 25 April 1996; revised 29 August 1996; accepted 6 September 1996.