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 *dhIA* 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–C₁₀. 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 (DhIA) from *Xanthobacter autotrophicus* GJ10 has been extensively studied and the sequence of the *dhIA* 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-

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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 halido-hydrolases. The first 17 residues of the N-terminal amino acid sequence were identical to those reported for halido-hydrolases 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 halido-hydrolase 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* DH5 α (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 Δ 20 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 μ g ml⁻¹), tetracycline (15 μ g ml⁻¹), IPTG (50 μ g ml⁻¹) and X-Gal (50 μ g 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 YEP 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 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.

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 *Sau*3A. Fragments of 15–25 kbp were isolated and ligated with cosmid vector pLAFR5 previously digested with *Bam*HI and *Sca*I. The resultant preparations were packaged into phage λ heads using an *in vitro* packaging kit (Amersham) and transduced into *E. coli* DH5 α 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 μ m). 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

Organism (plasmid)	Specific activity (nmol min ⁻¹ mg ⁻¹)*							
	ClC ₃	ClC ₄	ClC ₆	ClC ₈	ClC ₉	ClC ₁₀	ClC ₁₆	DCE
<i>R. rhodochrous</i> NCIMB 13064(pRTL1, pRTL2)	40	80	72	58	16	20	5	< 1
<i>E. coli</i>								
DH5 α (pLTL1k)	7	32	28	24	11	10	< 1	3
DH5 α (pUTL11)	ND	120	112	60	43	32	< 1	10
DH5 α (pUTL11X)	38	115	ND	68	40	28	< 1	10
DH5 α (pUTL112)	< 1	< 1	< 1	< 1	ND	< 1	< 1	ND

* 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; ClC₆, 1-chlorohexane; ClC₈, 1-chlorooctane; ClC₉, 1-chlorononane; ClC₁₀, 1-chlorodecane; ClC₁₆, 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 *Bam*HI 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, *Eco*RI; RV, *Eco*RV; K, *Kpn*I; Sm, *Sma*I; X, *Xba*I; B, *Bam*HI; *dhaA*, haloalkane dehalogenase gene locus, with direction of transcription (arrow).

Most of the recombinant clones tested (23 out of 25) had plasmid inserts with an average size of 23 ± 5 kbp. To facilitate the screening procedure the dehalogenase activity of clones was measured after growth in chloride-free 2YT medium in the presence of 1-chlorobutane as described in Methods, and four clones out of 115 tested showed an increased chloride ion release. Restriction analysis of the corresponding recombinant plasmids (pLTL1k, pLTL52, pLTL23 and pLTL4) indicated a common region in the cloned DNA. Plasmid pLTL1k (insertion of 18.4 kbp) was chosen for further analysis. The 5.8 kbp *Bam*HI fragment containing the putative haloalkane dehalogenase gene (*dhaA*) was sub-cloned from this plasmid into the pUC129 vector, and the resulting plasmid was designated pUTL11. When transformed with this plasmid, *E. coli* DH5 α expressed higher dehalogenase activities than detected in the original Dha⁺ clones (Table 1). Restriction mapping and further sub-cloning resulted in the localization of the *dhaA* gene within the 2 kbp DNA fragment of plasmid pUTL11X (Fig. 1).

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

plasmids pUTL11 and its shortened derivative pUTL11X (Table 1, and Fig. 1).

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,

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).

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 kbp *Xba*I–*Bam*HI 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.

DHAA	MSEIGTG-----FPFDPHYVEVL----GERMHYVDVGPRDGTPL	50
DEHH1	M-----DFP---GFKNSTVTVDGVDIAYTVSG--EGPPVL	50
DHLA	MINAIRTDPQRFNSLDQYFSPNYLDDLPGYPGLRAHYLDEGNSDAEDVF	50
	* *	
DHAA	F-LHGNPTSSYLWRNIIPHVA-PSHRCIAPDLIGMGKSDKP-----DLDY	100
DEHH1	M-LHGFPQNRAMWARVAPQLAEH-HTVVCADLRGYGDSKPKCLPDRSNY	100
DHLA	LCLHGEPWTSYLYRKMIPVFAESGARVIAPDFFGFGKSDKP---VDEEDY	100
	*** * * * * * * *	
DHAA	FFDDHVRYLDAFIEALGLEEVVLVIHDWGSALGFHWAKRNPVERVKGIACM	150
DEHH1	SFRTFAHDQLCVMRHLGFERFHLVGHDRGGRTGHRMALDHPEAVLSLTVM	150
DHLA	TFEFHRNLLALIERLRLNITLVVQDWGGFLGLTLPMDPSRFKRLIIM	150
	* * * *	
DHAA	E--FIRIPTWDEWPEFARETQAFRTA-----DVGRELIIDQNAFIE	200
DEHH1	DI-----VPTYAMFMNTNRLVAASYWHWYFLQOPEPFPEHMIGQDPDFY	200
DHLA	NACIMTDPVTPQAFSAFVTQPADGFTAW-----KYD---LVTPSDLRL	200
	* *	
DHAA	GALPKCVVRPLTEVEMDHYREFLKPVDREPLWRFPNELPIAGE---PAN	250
DEHH1	ETCLFGWGAT---KVSDFDQQLNA-YRES-WRNPAMIHGSCSDYRAAA	250
DHLA	<u>DQFMKRWAPLTAEASAYAAPFPDTSYQAGVRKFKPMVAQRDQ</u> ---ACI	250
	 *	
DHAA	IVALVEAYMNLWHSQVPVKLLFWGTGVLIPPAEAAARL-AESLPNC-KTV	300
DEHH1	TIDLEHDSADIQRKECPTLVFYGSKGQM---GQLFDIPAEWAKRCNNTT	300
DHLA	DIS-TEAISFWQNDWNGQTMAIGMKDKLLGPDVMYPM-KALINGCPEPL	300
	 *	
DHAA	DIG-PGLHYLQEDNPDLIGSEIARWLPLAL-	330
DEHH1	NASLPGGHFFVDQFAETSEILKFLARNG	330
DHLA	EIA-DAGHFVQE-FGEQVAREALKHFAETE	330
	 *	

Fig. 3. Amino acid sequence alignment of haloalkane dehalogenases from *R. rhodochrous* NCIMB 13064 (DHAA) and *X. autotrophicus* GJ10 (DHLA), and the haloacetate dehalogenase from *Moraxella* sp. (DEHH1). Catalytic residues of DHLA (Asp124, Trp125, Trp175, Asp260 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.

Previously we reported that the P200 Dha⁻ derivative of NCIMB 13064, which had lost a 20 kbp fragment of pRTL1, could revert to the original Dha⁺ phenotype (revertants were detected in cell populations at a frequency of approximately 10⁻⁷) with the restoration of pRTL1 (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⁻ mutant (P400) was isolated which had lost both pRTL1 and pRTL2. Surprisingly, this strain was also able to revert to the Dha⁺ phenotype at a frequency of about 10⁻⁷–10⁻⁸, with restoration of the original plasmids. It was therefore of interest to determine the state of the *dhaA* locus in these derivatives. To do this, total DNA preparations from strains P200 and P400 were hybridized with the *EcoRI*–*Bam*HI (nt 1178–2118) fragment of pUTL11X, using varying conditions of stringency (i.e. temperatures of hybridization from 40 °C to 65 °C with 5 °C intervals) and times of hybridization (from 4 to 18 h). The results presented in Fig. 4 were obtained when hybridization with this probe was carried out at 50 °C for 6 h. This probe gave one band when hybridized with total DNA *Bam*HI digests of Dha⁺ NCIMB 13064 and P210 (a Dha⁺ revertant of P200), but several bands of hybridization were detected with *Bam*HI digests of Dha⁻ strains P200 and P400. There was no difference in the hybridization patterns of strains P200 and P400. Four intense signals were in the region of fragments with a lower molecular mass than that of the parent NCIMB 13064. Similar results were obtained when the *XbaI*–*Bam*HI 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 micro-organisms 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

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 *Bam*HI, separated in 0.9% agarose gel and transferred onto Z-probe membrane. Hybridization was performed with the ³²P-labelled *Eco*RI-*Bam*HI fragment isolated from pUTL11X (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. 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 (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 DehH1 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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REFERENCES

- Birch, A., Häusler, A., Vögtli, M., Krek, W. & Hütter, R. (1989). Extremely large chromosomal deletions are intimately involved in genetic instability and genomic rearrangements in *Streptomyces glaucescens*. *Mol Gen Genet* 217, 447–458.
- Bradford, M. M. (1976). A rapid and sensitive detection method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal Biochem* 72, 248–254.
- Carafa, Y. A., Brody, E. & Thermes, C. (1990). Prediction of rho-independent *Escherichia coli* transcription terminators. *J Mol Biol* 216, 835–858.

- Curragh, H., Flynn, O., Larkin, M. J., Stafford, T. M., Hamilton, J. T. G. & Harper, D. B. (1994). Haloalkane degradation and assimilation by *Rhodococcus rhodochrous* NCIMB 13064. *Microbiology* 140, 1433–1442.
- DasGupta, U., Weston, H. K. & Berg, D. E. (1987). Local DNA sequence control of deletion formation in *Escherichia coli* plasmid pBR322. *Genetics* 115, 41–49.
- Flett, F. & Cullum, J. (1987). DNA deletions in spontaneous chloramphenicol-sensitive mutants of *Streptomyces coelicolor* A3(2) and *Streptomyces lividans* 66. *Mol Gen Genet* 207, 499–502.
- Franken, S. M., Rozenboom, H. J., Kalk, K. H. & Dijkstra, B. W. (1991). Crystal structure of haloalkane dehalogenase: an enzyme to deoxygenate halogenated alkanes. *EMBO J* 10, 1297–1302.
- Higgins, D. G. & Sharp, P. M. (1988). CLUSTAL: a package for performing multiple sequence alignments on a microcomputer. *Gene* 73, 237–244.
- Janssen, D. B., Gerritse, J., Brakman, J., Kalk, C., Jager, D. & Witholt, B. (1988). Purification and characterisation of a bacterial dehalogenase with activity towards halogenated alkanes, alcohols and ethers. *Eur J Biochem* 171, 67–72.
- Janssen, D. B., Pries, P., Vander Ploeg, J., Kazemier, B. & Terpstra, P. (1989). Cloning of 1,2-dichloroethane degradation genes of *Xanthobacter autotrophicus* GJ10 and expression and sequencing of the *dhlA* gene. *J Bacteriol* 171, 6791–6799.
- Kawasaki, H., Tsuda, K., Matsushita, I. & Tonomura, K. (1992). Lack of homology between two haloacetate dehalogenase genes encoded on a plasmid from *Moraxella* sp. strain B. *J Gen Microbiol* 138, 1317–1323.
- Keen, N. T., Tamaki, S., Kobayashi, D. & Trollinger, D. (1988). Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. *Gene* 70, 191–197.
- Keuning, S., Janssen, D. B. & Witholt, B. (1985). Purification and characterisation of a hydrolytic haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10. *J Bacteriol* 163, 635–639.
- Kulakova, A. N., Stafford, T. M., Larkin, M. J. & Kulakov, L. A. (1995). Plasmid pRTL1 controlling 1-chloroalkane degradation by *Rhodococcus rhodochrous* NCIMB 13064. *Plasmid* 33, 208–217.
- Lopez, P., Espinosa, M., Greeberg, B. & Lacks, S. A. (1984). Generation of deletions on pneumococcal *mal* genes cloned in *Bacillus subtilis*. *Proc Natl Acad Sci USA* 81, 5189–5193.
- McCorkle, G. & Altman, S. (1982). Large deletion mutants of *Escherichia coli* tRNA^{tyr}. *J Mol Biol* 155, 83–103.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Pearson, W. R. & Lipman, D. J. (1988). Improved tools for biological sequence comparison. *Proc Natl Acad Sci USA* 85, 2444–2448.
- Peeters, B. P. H., de Boer, J. H., Bron, S. & Venema, G. (1988). Structural plasmid instability in *Bacillus subtilis*: effect of direct and inverted repeats. *Mol Gen Genet* 212, 450–458.
- Pries, F. (1995). *Protein engineering of haloalkane dehalogenase*. PhD thesis, Rijksuniversiteit Groningen.
- Pries, F., Kingma, J., Pentenga, M., Van Pouderooyen, G., Jeronimus-Stratingh, C. M., Bruins, A. P. & Janssen, D. B. (1994). Site directed mutagenesis and oxygen isotope incorporation studies of the nucleophilic aspartate of haloalkane dehalogenase. *Biochemistry* 33, 1242–1247.
- Pries, F., Kingma, J., Krooshof, G. H., Jeronimus-Stratingh, C. M., Bruins, A. P. & Janssen, D. B. (1995). Histidine289 is essential for hydrolysis of the alkyl-enzyme intermediate of haloalkane dehalogenase. *J Biol Chem* 270, 10405–10411.
- Sallis, P. J., Armfield, S. J., Bull, A. F. & Hardman, D. H. (1990). Isolation and characterisation of halidohydrolase from *Rhodococcus erythropolis* Y2. *J Gen Microbiol* 136, 115–120.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schneider, D., Aigle, B., Leblond, P., Simonet, J.-M. & Decaris, B. (1993). Analysis of genome instability in *Streptomyces ambofaciens*. *J Gen Microbiol* 139, 2559–2567.
- Scholtz, R., Leisinger, T., Suter, F. & Cook, A. M. (1987). Characterisation of 1-chlorohexane halidohydrolase, a dehalogenase of a wide substrate range from *Arthrobacter* sp. *J Bacteriol* 169, 5016–5021.
- Schreiner, A., Fochs, K., Lottspeich, F., Poth, H. & Lingens, F. (1991). Degradation of 2-methylaniline in *Rhodococcus rhodochrous*: cloning and expression of two clustered catechol 2,3-dioxygenase genes from strain CTM. *J Gen Microbiol* 137, 2041–2048.
- Shine, J. & Dalgarno, L. (1974). The 3'-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to non-sense triplets and ribosomal binding sites. *Proc Natl Acad Sci USA* 71, 1342–1346.
- Slater, J. H., Weightman, A. J. & Hall, B. G. (1985). Dehalogenase genes of *Pseudomonas putida* PP3 on chromosomally located transposable elements. *Mol Biol Evol* 2, 557–567.
- Stafford, T. M. (1993). *Haloalkane degradation by Rhodococcus rhodochrous* NCIMB 13064. PhD thesis, The Queen's University of Belfast.
- Tardif, G., Greer, C. W., Labbe, D. & Lan, P. C. K. (1991). Involvement of a large plasmid in the degradation of 1,2-dichloroethane by *Xanthobacter autotrophicus*. *Appl Environ Microbiol* 57, 1853–1857.
- Thomas, A. W., Slater, J. H. & Weightman, A. J. (1992a). The dehalogenase gene *dhl* from *Pseudomonas putida* PP3 is carried on an unusual mobile genetic element designated DEH. *J Bacteriol* 174, 1932–1940.
- Thomas, A. W., Topping, A. W., Slater, J. H. & Weightman, A. J. (1992b). Localisation and functional analysis of structural and regulatory dehalogenase genes carried on DEH from *Pseudomonas putida* PP3. *J Bacteriol* 174, 1941–1947.
- Verschueren, K. H. G., Franken, S. M., Rozenboom, H. J., Kalk, K. H. & Dijkstra, B. W. (1993). Refined X-ray structures of haloalkane dehalogenase at pH 6.2 and pH 8.2 and implications for the reaction mechanism. *J Mol Biol* 232, 856–872.
- Yokota, T., Omori, T. & Kodama, T. (1987). Purification and properties of haloalkane dehalogenase from *Corynebacterium* sp. strain m15-3. *J Bacteriol* 169, 4049–4054.

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