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The nucleotide sequence of a transposable haloalkanoic acid dehalogenase regulatory gene (*dehR$_I$*) from *Pseudomonas putida* strain PP3 and its relationship with σ^{54}-dependent activators

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Abstract

The mobile genetic element, *DEH* found in *Pseudomonas putida* PP3 carries a 2-haloalkanoic acid dehalogenase structural gene, *dehI*, and its associated regulatory gene, *dehR$_I$*. The nucleotide sequence of *dehR$_I$* was determined. The gene had an open reading frame putatively encoding for a 64 kDa protein containing 571 amino acid residues. The protein was similar to previously published sequences of several other σ^{54}-dependent activator proteins. Amino acid sequence analysis showed that the deduced DehR$_I$ protein clustered with the NifA nitrogenase regulatory activator family, and was most closely related, with 47.7% similarity, to a 'NifA-like' deduced partial sequence from a plasmid-encoded ORF in *Pseudomonas* sp. strain NS671, associated with L-amino acid production. The domain structure of DehR$_I$ was analysed by alignment with other NifA-like and NtrC-like sequences and showed a highly conserved central region of approximately 230 amino acids, and a potential DNA-binding domain. No homology was detected between the deduced DehR$_I$ and other σ^{54}-dependent activator sequences at the N-terminus, a result which was consistent with that region being the domain which recognised inducer.

Introduction

Pseudomonas putida strain PP3 was isolated from a microbial community growing on the herbicide Dalapon (2,2-dichloropropionic acid – 22DCPA) (Senior et al. 1976). *P. putida* PP3 produced two different 2-haloalkanoic acid dehalogenases, DehI and DehII, which catalysed the hydrolytic removal of halogens from compounds, such as 22DCPA, and related substrates, such as 2-monochloropropionic acid (2MCPA) (Slater et al. 1979; Weightman et al. 1979). Slater et al. (1985) suggested that the genes encoding DehI and DehII, namely *dehI* and *dehII*, were carried on one or more transposons. Thomas et al. (1992a) confirmed that *dehI* was present on an unusual mobile genetic element, *DEH*, which inserted at a high frequencies into target plasmids, such as pWW0 and RP4.5. When hybrid plasmids containing *dehI* were transferred to other *Pseudomonas putida, P. aeruginosa* and *Escherichia coli* strains, *dehI* was regulated in a manner identical to the parental strain. These and other results indicated that *DEH* carried a regulatory gene, designated *dehR$_I$*, as well as *dehI*.

Thomas et al. (1992b) mapped an 11.6-kb *Eco*RI-G restriction fragment from a derivative of a TOL plasmid containing the dehalogenase transposon, pWW0::*DEH*. The position of *dehI* was determined and an adjacent activator gene which regulated *dehI* expression was identified by transposon mutagenesis and complementation analysis (Thomas et al. 1992b) (see Fig. 1). Expression of *dehI* was dependent on the presence of a functional σ^{54} polymerase, since it was not expressed in *rpoN* mutants of *P. putida* (Thomas et al. 1992b). A promoter motif of the -12/-24 type was identified upstream from the *dehI* initiation codon (Thomas 1990; Topping 1992). These promoters are recognized by σ^{54} family of sigma factors associated with RNA polymerases involved with the expression

Fig. 1. A physical map of part of the transposon, *DEH*, showing the relationship between the 2-haloalkanoic acid dehalogenase gene, *dehI*, and its adjacent regulator gene, $dehR_I$. The arrows indicate the direction of transcription deduced from the promoter motifs located within a control region designated C. The distances indicated are in kb and are related to a key *Pst*I site located within the complete transposon (see Fig. 2).

of a variety of metabolic functions in Gram-positive and Gram-negative bacteria (Dixon 1986; Thöny & Hennecke 1989; Merrick 1993). Little is know about the regulation of dehalogenase genes, and this is the first report describing a dehalogenase gene transcribed from a σ^{54}-dependent promoter.

The detailed analysis of the nucleotide sequence of $dehR_I$ described in this paper provides further evidence that the dehalogenase regulatory system is related to other σ^{54}-dependent activators. The domain structure of the deduced $DehR_I$ amino acid sequence is also reported and shows similarities with a number of NifA-like activators.

Materials and methods

Bacterial strains and plasmids

Pseudomonas putida strain PP3 (DCA^S, $2mcpa^+$, $DehI^+$, $DehII^+$) was isolated from a microbial community growing on the herbicide Dalapon (2,2-dichloropropionic acid, 22DCPA) (Senior et al. 1976), and grown as previously described (Slater et al. 1979). *Escherichia coli* strain JM107 (Yanish-Peron et al. 1985) was used as the host for all recombinant plasmids and grown as previously described (Thomas et al. 1992a, b).

Plasmid pAWT6, derived from pHG327, contained the *Eco*RI-G restriction fragment of TOL plasmid pWW0 into which *DEH* had inserted naturally (Thomas et al. 1992b). Plasmid pAWT43 was the vector plasmid pBTac1 (Boehringer Mannheim, Lewes, UK) with a 4.2-kb *Hin*dIII restriction fragment insert from pAWT6 which overexpressed of *dehI* under *tac* promoter control. M13mp18 phage and plasmid pUC18 (see Sambrook et al. 1989) were prepared from *E. coli* JM107.

DNA template preparation and DNA sequencing

Restriction fragments of plasmid pAWT6 were isolated from agarose gels using GeneClean II (Stratech, Luton, UK) and the manufacturer's protocol, and sequenced by both manual and automated procedures. Fragments were sonicated for 2 × 60 sec with cooling in a Heat Systems W375 sonicator at full power and 42% duty cycle (Dr M M Binns at The Animal Health Trust, Newmarket, Suffolk, UK). Klenow fragment of DNA polymerase I (Boehringer Mannheim) and T7 DNA polymerase (Boehringer Mannheim) were used to repair the sonicated fragments (Sambrook et al. 1989). The fragments were separated on a 1% (w v^{-1}) agarose gel and fragments in the range 600 to 900 bp eluted from the gel with GeneClean II. Fragments were either cloned directly or via *Eco*RI linkers into M13mp18 phage or pUC18 plasmid using standard procedures (Sambrook et al. 1989). DNA templates were prepared for sequencing using a Pharmacia Miniprep Plus kit (Pharmacia UK), a phenol/chloroform extraction and purification with GeneCleanII, resin according to manufacturer's protocol (Stratech). Single stranded DNA was isolated using the polyethylene glycol (PEG) 8000 phage precipitation technique (Sambrook et al. 1989).

DNA templates were sequenced manually using a Sequenase II kit (Amersham International, Amersham, Bucks, UK) with ^{35}S-dATP label (Amersham International) and universal or reverse M13 primers, using the manufacturer's protocol. Electrophoresis was carried out at 1500 W (constant power) in 6% (w v^{-1}) polyacrylamide gel incorporating 7 M urea. After electrophoresis the gel was transferred to 3M filter paper (Whatman, Maidstone, Kent, UK) and dried slowly under vacuum. The dried gel was exposed to X-ray film (Fuji, London, UK) at room temperature.

Sequencing reactions with ALF automated DNA sequencer (Pharmacia UK) were carried out using a cycle sequencing protocol (Dr T Hawkins, MRC Laboratory, Cambridge, UK, personal communication).

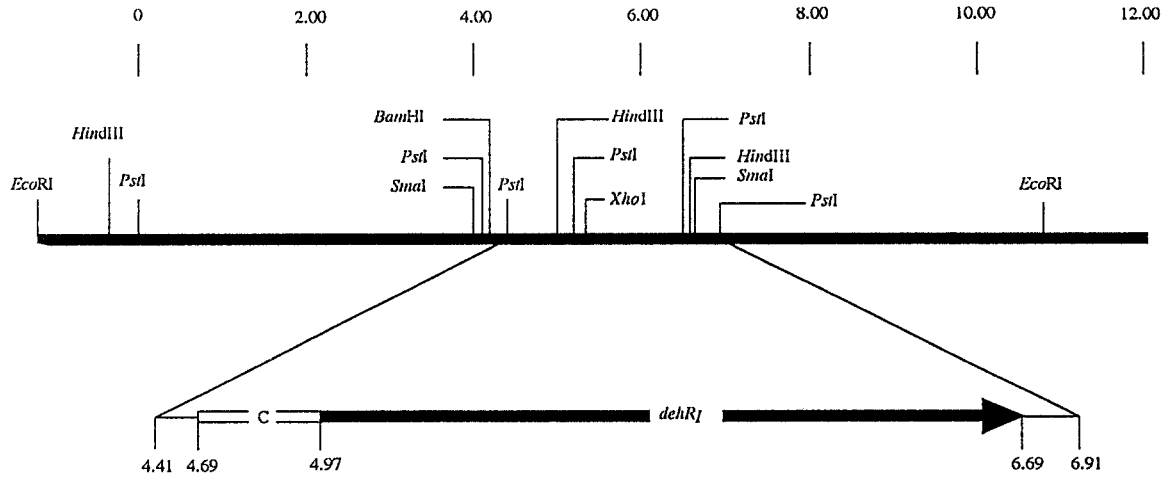

Fig. 2. A detailed physical map showing the detailed arrangement of the regulator gene, $dehR_I$, and the key restriction sites within transposon *DEH*.

The reaction mixtures of 7 μl contained: 60 ng double-stranded template DNA; 70 mM Tris-HCl pH 8.9; 16 mM $(NH_4)_2SO_4$; 5 mM $MgCl_2$; 7% (v v^{-1}) DMSO; 0.25 pmoles fluorescein labelled primer; 0.5 units *Taq* DNA polymerase (Boehringer Mannheim, Lewes, Sussex, UK), and deoxynucleotides at final concentrations of 40 μM each, except the 'stop' deoxynucleotide which was added at 10 μM. Dideoxynucleotides were added to the following concentrations: ddATP 240 μM; ddCTP 100μM; ddGTP 20 μM; ddTTP 200 μM. The sequence cycle conditions were: denaturation at 93° C for 30 sec, primer annealing at either 55° C for the M13 universal primer or 49° C for the M13 reverse primer for 30 sec, and extention at 70° C for 72 sec. These cycles were repeated 20 times followed by 6 cycles with denaturation at 93° C for 30 sec and extention for 150 sec. When the reactions were complete, 5 μl formamide (50% v v^{-1}) in blue dextran was added to each tube and the stopped reaction mixtures taken from under the oil to a fresh tube. Sample loading, electrophoresis and sequence reading using ALF apparatus was carried out following manufacturer's instructions (Pharmacia UK).

Synthesis of sequencing primers

A single concensus sequence was derived from the individually sequenced sonicated fragments. Any gaps or under-represented regions were re-sequenced using *DEH* specific oligonucleotide primers. These primers were manufactured on a PCR-Mate DNA synthesiser (Applied Biosystems Ltd., Warrington, UK) with the addition a fluorescein phosphoamidite (Pharmacia UK) to allow use of the ALF equipment. When synthesis was completed the oligonucleotides were flushed from the column using fresh, concentrated NH_3 solution. Deprotection of oligonucleotides was carried out overnight at 56° C in the NH_3 solution. Purification was performed using a NAP-10 column (Pharmacia UK) in accordance with the manufacturer's instructions. The concentration of the eluted solution was determined from the absorbance at 260 nm and adjusted to 0.14 A_{260} unit ml^{-1}.

Sequence analysis

Sequencing data were connected into a single concensus sequence using the DNASIS computer program (Hitachi, Japan). Translations and homology searches were carried out using the UWGCG and PHYLIP software available on the SEQNET computer (BBSRC, Daresbury, UK). The conserved central region of the derived $DehR_I$ amino-acid sequence (Fig. 2) was aligned with sequences of other σ^{54}-dependent activators selected from the GenEMBL and SWISSPROT databases, using the CLUSTALV package (Higgins et al. 1992). The alignment was edited to remove all but the conserved regions and analysed by Neighbor-Joining programmes in the PHYLIP (Felsenstein 1989) package.

-35 -10

CCGCGCAAACCATATTTGACCCTGACTGTCAATTGCGCCTATGGTTAATCAATAAGTCAC

M R M S K S

60 CAAAAGAGCGCACCCACAACAAAGCAATCTCTTAAAGGAAATATGAGGATGAGCAAATCA

T Y D V L S T V L D A L P I G V V E V H

120 ACCTATGACGTTCTTAGCACCGTCCTGGATGCATTACCGATTGGTGTTGTGGAAGTGCAT

G D T I L A M N R V A R S L F G G R Y D

180 GGTGACACGATCTTGGCAATGAATCGCGTCGCCCGAAGCTTGTTTGGCGGCCGGTACGAT

S L A Q L E Q E N A L M V S M I T T E P

240 TCATTGGCGCAACTAGAGCAGGAAAATGCATTGATGGTGAGCATGATCACGACAGAACCG

N N G N R W V N I D G A I Y Y R D Q L P

300 AACAATGGTAACCGGTGGGTAAATATTGATGGCGCGATCTATTACCGCGATCAACTGCCC

S D E G K V T L L V P V A Y M D L R N P

360 TCTGATGAGGGAAAGGTCACCCTGCTTGTTCCCGTGGCCTACATGGACTTGCGCAATCCA

E L Q E L R Q K Y E D F L E I F H N C Y

420 GAACTGCAGGAATTGCGGCAGAAATACGAGGATTTCCTAGAGATTTTTCACAATTGCTAC

D G I Y V A D G R G K T L W L N E G F E

480 GATGGCATCTATGTCGCCGACGGCAGAGGTAAAACCTTATGGCTGAACGAGGGATTCGAG

R A Y G V S R D Q F I G Q D A R E L E R

540 CGTGCTTACGGTGTCAGTCGGGACCAGTTTATTGGCCAAGATGCGAGAGAACTCGAGCGT

R G Y A K P L I T W R V I S T G K R I T

600 CGGGGCTACGCTAAGCCACTCATTACATGGCGAGTCATATCGACCGGAAAACGGATCACG

V V H K T N T G K S V L A T G I P L F D

660 GTGGTCCATAAAACCAATACCGGCAAAAGTGTGCTCGCGACCGGCATACCGCTTTTCGAC

E S G K V R K V I V N S R D M T E L H Q

720 GAAAGCGGGAAGGTGCGAAAAGTGATTGTCAATTCACGCGACATGACTGAGTTGCATCAG

L R E Q L N Q A E K N I A R Y E S E L E

Fig. 3.

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780   CTACGCGAGCAGCTCAATCAGGCTGAAAAAAATATCGCCCGCTACGAGTCCGAACTGGAG
        R  L  Q  T  R  N  A  T  C  R  L  V  D  S  S  G  K  A  T  R
840   CGGCTGCAGACGCGAAACGCAACGTGCAGATTGGTGGATTCGTCTGGAAAAGCGACAAGA
        C  A  T  S  Y  T  W  R  T  R  L  A  K  V  D  T  A  L  L  I
900   TGCGCGACGTCTTACACTTGGCGCACACGACTGGCCAAGGTCGATACGGCATTACTCATC
        T  G  E  S  G  V  G  K  E  V  I  A  K  L  V  H  N  E  S  D
960   ACCGGTGAATCCGGCGTGGGAAAAGAAGTGATCGCCAAGCTGGTACACAATGAAAGTGAC
        R  K  E  G  R  L  I  K  I  N  C  G  A  I  P  E  Q  L  L  E
1020  CGAAAGGAAGGCCGGCTAATCAAGATCAATTGTGGCGCCATTCCTGAACAATTGCTTGAG
        S  E  L  F  G  Y  E  K  G  A  F  T  G  S  N  K  Q  G  K  P
1080  TCGGAACTGTTTGGCTATGAAAAAGGCGCATTTACCGGATCGAATAAGCAGGGAAAGCCT
        G  L  L  E  L  A  D  K  G  T  L  F  L  D  E  I  G  E  M  P
1140  GGCCTGCTTGAGCTCGCTGACAAGGGAACGCTTTTTCTAGATGAAATCGGCGAGATGCCG
        L  D  L  Q  V  K  L  L  Q  V  L  Q  D  K  T  F  T  R  V  G
1200  TTGGACCTTCAAGTCAAACTGTTACAGGTCTTGCAGGATAAAACCTTCACTCGTGTTGGC
        G  T  I  T  V  H  V  D  F  R  V  I  A  A  T  N  R  D  L  E
1260  GGCACCATTACGGTCCATGTTGATTTTCGGGTAATTGCAGCCACGAATCGCGATCTGGAA
        D  V  S  A  R  A  T  F  R  E  D  L  F  Y  R  L  S  V  V  P
1320  GATGTTAGTGCGCGCGCGACCTTCAGAGAAGATCTTTTTTATCGCCTAAGCGTAGTGCCA
        L  K  V  P  P  L  R  E  R  Q  E  D  V  V  P  L  L  E  H  F
1380  CTGAAAGTCCCGCCTCTGCGGGAGCGGCAAGAAGACGTTGTTCCACTTCTGGAGCATTTC
        L  A  E  F  N  K  R  H  H  F  S  K  R  F  S  E  G  V  M  Q
1440  CTCGCCGAGTTTAACAAGCGCCATCACTTTTCTAAGCGATTTTCCGAAGGCGTCATGCAG
        Q  L  L  E  H  S  W  P  G  N  V  R  E  L  R  N  L  V  E  R
1500  CAGCTTTTGGAGCACTCATGGCCGGGCAATGTGAGAGAGCTGCGCAACCTCGTGGAACGT
        L  V  V  I  S  P  T  D  I  I  G  T  N  S  L  P  E  K  L  A
1560  TTAGTGGTAATTTCACCCACCGATATCATTGGCACAAACTCACTTCCGGAGAAGCTAGCG
        P  G  F  S  E  D  F  S  A  G  L  D  F  Q  A  A  V  A  A  Y
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Fig. 3. Continued.

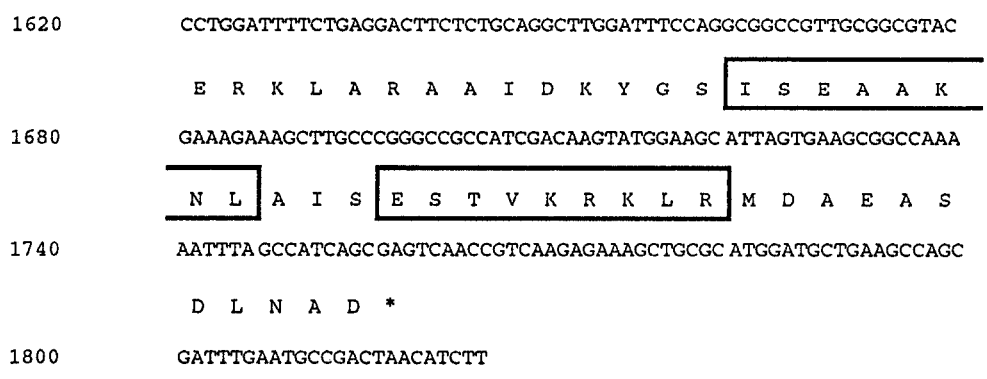

Fig. 3. Nucleotide and deduced amino acid sequences of *dehR*$_I$. The putative -35/-10 promoter is indicated with the relevant bases underlined. The double-boxed residues indicate the conserved central region, corresponding to the domain-C of Thöny & Hennecke (1989). The boxed residues indicate a putative helix-turn-helix motif at the C-terminus of DehR$_I$.

Results and discussion

The DNA sequenced in this study was cloned from plasmid pAWT5 (= pWWO::*DEH*) (Thomas et al. 1992a). Figure 2 shows the location of *dehR*$_I$ relative to *dehI* on *DEH* as mapped by Thomas et al. (1992b). The data shown in Fig. 3 was obtained from sequencing of the central *Sma*I fragment from 4007 bp to 6564 bp, and the overlapping *Pst*I fragment from 4409 bp to 6910 bp in Fig. 2.

Figure 3 shows the open reading frame from base position 102 whose location and size suggested that this was the dehalogenase regulatory gene *dehR*$_I$. Complementation analysis and results from transposon mutagenesis reported previously were consistent with this region encoding a positive regulatory element (Thomas et al. 1992b). The 1716 bases of the open reading frame coded for a putative protein of 571 amino-acid residues and a calculated molecular weight of 64 kDa. Analysis of the DNA sequence flanking the *dehR*$_I$ identified a highly conserved -35/-10 type promoter motif about 60 bases upstream from the putative translational start of DehR$_I$ (Fig. 3).

An alignment of the derived amino-acid sequence of DehR$_I$ with other σ^{54}-dependent activators clearly showed that the carboxy- and amino-terminal domains were divergent, whilst a central domain of approximately 230 amino acids was considerably conserved. It has been proposed that this central region, designated domain-C by Drummond et al. (1986), is responsible for interactions with the RNA polymerase and ATP binding (Thöny & Hennecke 1989; Huala & Ausubel 1989; Kustu et al. 1991; North et al. 1993). A range of genes in a variety of bacteria are known to be controlled by similar proteins, and the similarity of some activators has been shown to allow cross activation. Two other σ^{54}-dependent catabolic gene activators, DmpR, which regulates the *dmp* operon associated with dimethylphenol catabolism in *P. putida* (Shingler et al. 1993; Fernandez et al. 1994), and XylR which regulates the TOL plasmid *xyl*-regulon in *P. putida* (de Lorenzo et al. 1991), have 67% deduced amino acid similarity over their entire lengths and > 79% in the conserved central domain. Fernandez et al. (1994) constructed recombinant organisms in which *xyl* catabolic genes were activated by DmpR, and *dmp* catabolic genes were activated by XylR, thus demonstrating that these two proteins were also functionally conserved. However, plasmids which expressed NtrC and NifA constitutively (kindly supplied by Dr R. Dixon) were not able to activate *dehI* expression when placed in *trans* with a plasmid construct containing intact *dehI* and its adjacent control region (A.W. Topping, unpublished observations).

Figure 4 shows a cladogram illustrating the relationships between a range of σ^{54}-dependent activators. The analysis used to construct the cladogram was based on an alignment of amino acids in the conserved central domain highlighted in Fig. 3. It indicates that DehR$_I$ is not closely related to XylR (25.6% similarity over 229 residues in the C-domain) or DmpR (26.1% similarity over 229

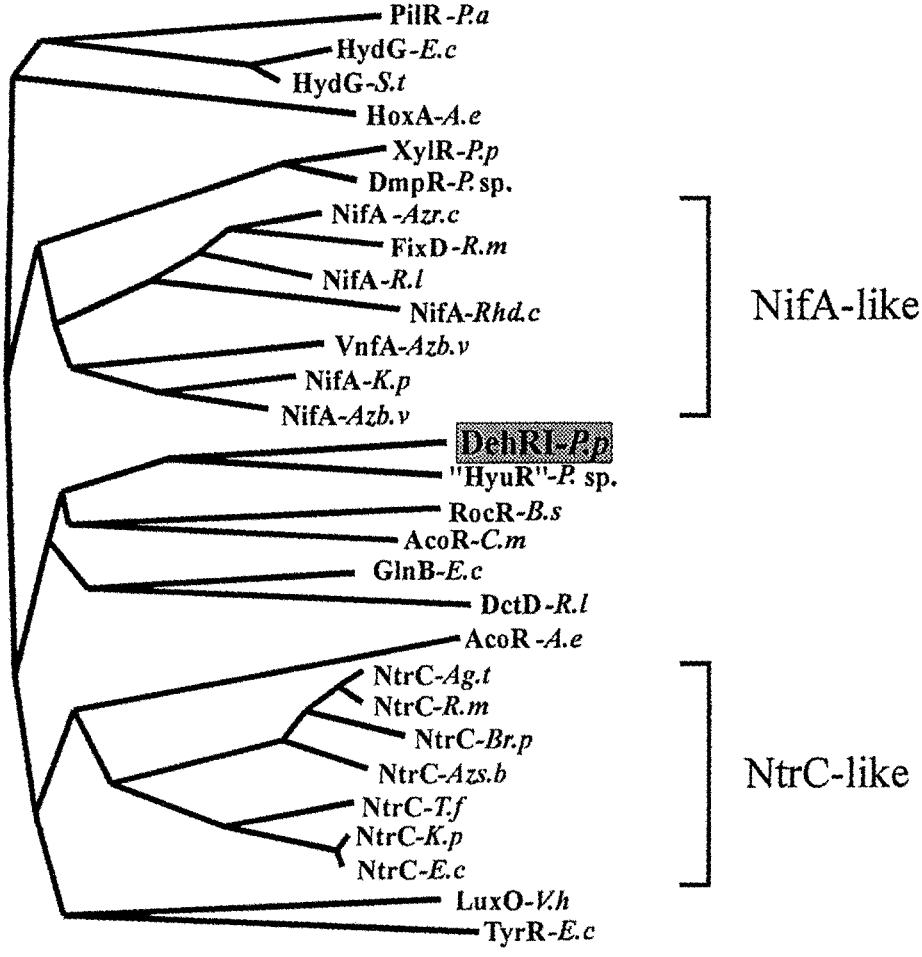

Fig. 4. A cladogram illustrating the relationships between the derived $DehR_I$ amino-acid sequence and those of other σ^{54}-dependent activators. The cladogram was produced after an alignment of the conserved central regions (see text) of the selected activators using CLUSTALV (Higgins et al. 1992) and manual editing. Sequence similarities were determined by the Dayhoff PAM matrix method and Neighbor-Joining using the PHYLIP package (Felsenstein 1989).The following sequences were obtained from the GenBank database unless noted: AcoR-*A.e*; acetoin catabolic regulator from *Alcaligenes eutrophus* (P28614 – SWISSPROT database); AcoR-*C.m*, acetoin catabolic regulator from *Clostridium magnum* (L31844); DctD-*R.l*, C4-dicarboxylate transport regulator from *Rhizobium leguminosarum* (X06253); DehRI-*P.p*, dehalogenase regulator from *Pseudomonas putida* (U23716); DmpR-*P.* sp., dimethylphenol catabolic regulator from *Pseudomonas* sp. CF600 (X68033); FixD-*R.m*, nitrogenase regulator from *Rhizobium meloloti* (X03065); GlnB-*E.c*, glutamine metabolic regulator from *Escherichia coli* (S67014); HoxA-*A.e*, hydrogenase regulator from *A. eutrophus* (M64593); HydG-*E.c*, hydrogenase regulator from *E. coli* (M28369); HydG-*S.t*, hydrogenase regulator from *Salmonella typhimurium* (M64988); 'HyuR'-*P.* sp., hydantoin catabolic regulator from *Pseudomonas* sp. NS671 (Q01265-SWISSPROT); LuxO-*V.h*, luminescence regulator from *Vibrio harveyi* (L26221); NifA-*Azb.v*, nitrogenase regulator from *Azotobacter vinelandii* (Y00554); NifA-*Azr.c*, nitrogenase regulator from *Azorhizobium caulinodans* (X08014); NifA-*K.p*, nitrogenase regulator from *Klebsiella pneumoniae* (X02616); NifA-*R.l*, nitrogenase regulator from *R. leguminosarum* (L11084); NifA-*Rhd.c*, nitrogenase regulator from *Rhodobacter capsulatus* (X07567); NtrC-*Ag.t*, nitrogen regulator from *Agrobacterium tumefaciens* (J03678); NtrC-*Azs.b*, nitrogen regulator from *Azospirillum brasilense* (X67684); NtrC-*Br.p*, nitrogen regulator from *Bradyrhizobium parasponia* (M14227); NtrC-*E.c*, nitrogen regulator form *E. coli* (X05173); NtrC-*K.p*, nitrogen regulator from *K. pneumoniae* (X02617); NtrC-*R.m*, nitrogen regulator from *R. meliloti* (M15810); NtrC-*T.f*, nitrogen regulator from *Thiobacillus ferrooxidans* (L18975); PilR-*P.a*, fimbriae expression regulator from *Pseudomonas aeruginosa* (Q00934-SWISSPROT); RocR-*B.s*, ornithine aminotransferase regulator from *Bacillus subtilis* (L22006); *Tyr-E.c*, tryptophan biosynthetic regulator from *E. coli* (M12114); VnfA-*Azb.v*, nitrogenase regulator from *A. vinelandii* (M26752); XylR-*P.p*, methylbenzoate catabolic regulator from *P. putida* (P06519-SWISSPROT).

residues in the C-domain) or DmpR (26.1% similarity over 229 residues in the C-domain), the only other σ^{54}-dependent xenobiotic-degrading gene activators whose derived amino-acid sequences have so far been reported. Indeed, according to this analysis $DehR_I$ did not cluster with either the NifA or NtrC groups. The most similar sequence identified from searches of databases was that derived from part of a putative regulatory protein encoded by a gene sequence (here referred to as *hyuR*) carried on a plasmid in *Pseudomonas* sp. strain. NS672, associated with L-amino acid biosynthesis (Watabe et al. 1992). Derived $DehR_I$ and HyuR sequences showed only 48% sequence similarity in the conserved central domain. Thus, whilst $DehR_I$ showed some sequence similarity to other σ^{54}-dependent activators, it was sufficiently different to suggest that it may represent a separate line of evolution. The similarity of $DehR_I$ to other activator proteins, together with previous results which showed DehI production to be dependent on the presence of σ^{54} and under positive control (Thomas et al. 1992b), led to a search for other features which might be associated with regulation by $DehR_I$. A DNA binding helix-turn-helix motif (Dodd & Egan 1990) was identified at the C-terminus, and is indicated in Fig. 3.

It is interesting to note that the G+C content of *dehR*$_I$, at 51.7% over the entire ORF, was significantly lower than expected for a *Pseudomonas* species which is reported to average 63% (Normore 1976). Although there are no other data available for dehalogenase regulatory genes in *Pseudomonas* spp., Murdiyato et al. (1992) and Jones et al. (1992) reported similarly low G + C contents for dehalogenase structural genes. Such inconsistencies between G + C content of dehalogenase genes and their hosts is consistent with acqusition of the genes by *Pseudomonas* species from other organisms in which they originated, a possibility which accords with the location of *dehR*$_I$ (and *dehI*) on a mobile genetic element (Thomas et al. 1992a).

The sequencing of *dehR*$_I$ has provided data on the mode of regulation of a 2-haloalkanoic acid dehalogenase. Although further work is required to confirm the biological activity of the identified features, these results provide useful information in this important area, and should stimulate work on the details of regulation in other dehalogenase systems.

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