REGULATION OF DEHALOGENASE E (DEHE) AND EXPRESSION OF DEHALOGENASE REGULATOR GENE (DEHR) FROM RHIZOBIUM SP. RC1 IN E. COLI

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ABSTRACT
The DNA sequence upstream of dehE gene encoding dehalogenase E (DehE) of Rhizobium sp. RC1 was determined and contained an open reading frame, designated dehR, which encoded a protein with a significant similarity to dehalogenase regulatory protein (DehR). Plasmid DNA designated pH648 that carry both dehE and dehR genes were cloned from Rhizobium sp. RC1 genomic DNA. The Rhizobium sp. RC1 genetic organization was determined, suggesting dehE was controlled by the product of dehR. Current study proved that by growth experiment, E. coli XL10 Gold::pFH648 (dehE+, dehR+) has the ability to grow in minimal media supplied with 20 mM D,L-2-chloropropionic acid (D,L-2CP) as sole source of carbon. E. coli XL10::pSC520 (dehE−) lacking dehR gene and E. coli XL10 Gold::pFH45 (dehR−) lacking dehE gene did not grow in minimal media supplied with 20 mM D,L-2CP as sole source of carbon and energy, suggesting both dehE and dehR genes were needed to allow growth in D,L-2CP minimal media. Since the genetic organisation for both dehE and dehR were neighbouring genes similar to that of Pseudomonas putida PP3 dehR, and dehI, promoters were predicted to be present for both dehE and dehR genes.


Keywords: biodegradation, regulator gene, bioremediation, dehalogenase regulator, promoter

Introduction
Halogenated organic compounds are the most prevalent class of environmental contaminants released into the environment as a result of their use in agriculture and industry (5, 23). Various types of haloalkanoic acid dehalogenases from different bacteria have been isolated and characterized (9, 14, 16, 17). Bacterial assimilation of these compounds is attributed to a group of enzymes called dehalogenases. These organisms can dehalogenate a wide range of chlorinated substrates via hydrolysis of the aliphatic carbon-halogen bond.

Previously, a soil bacterium isolated on 2,2-dichloro-propionic acid and identified as a Rhizobium sp. was found to produce three haloalkanoate dehalogenases (13). It was curious why this organism produced more than one dehalogenase when all three dehalogenases (DehD, DehE and DehL) could act on D,L-2-chloropropionic acid (D,L-2CP), with DehD being stereospecific for D-2-chloropropionic acid (D-2CP), DehL, being stereospecific for L-2-chloropropionic acid (L-2CP) and DehE acting on both stereoisomers. DehD and DehL acted collectively on monochloroacetic acid, dichloroacetic acid, 2-chlorobutyric acid and 2,3-dichloropropionic acid with DehE acting on all of these compounds (1, 13). It is a common feature that many soil bacteria produced more than one dehalogenase (7).

Expression of Rhizobium sp. RC1 dehalogenases is well regulated whereby, dehalogenase enzyme activity was not detected when Rhizobium sp. grown in non-halogenated compound such as lactate and pyruvate. Rhizobium sp. dehalogenases were also studied at a genetic level using a series of mutant strains (12). Mutant analysis had suggested that all three Rhizobium sp. dehalogenase genes were under the control of a single regulatory gene (dehR). The dehR was proposed to encode a regulator protein which controlled positively dehalogenase formation at the transcriptional level. A model was proposed in which the dehR product is an activator protein, which in the absence of inducer may not bind to the promoter region of the structural gene, and therefore transcription does not occur.

To date the relative location of dehD and dehL genes has been confirmed by sequence analysis (4). dehD was located upstream of dehL with 177 bp of non-coding region. The third Rhizobium sp. dehE gene was also sequenced. However, this gene is not particularly close to dehD and dehL and its relative location to them was not known. Upstream of dehE was found a second truncated ORF containing partial putative dehalogenase regulatory gene (dehR) of Rhizobium sp. RC1 (19). The partial deduced amino acid sequence showed a significant identity of 51% and similarity of 74% when conservative substitution were taken into account to the N-terminal region of a Pseudomonas putida dehalogenase regulatory gene product (22).

Currently, the function of gene regulation of dehalogenase gene expression in dehalogenase producing bacteria is not well studied. As a first step in establishing whether a single regulator
gene (dehR) controls dehE gene, we have cloned both genes together and demonstrates that E. coli acquiring both dehE and dehR is able to grow in minimal media supplied with D,L-2CP as sole source of carbon and energy.

Materials and Methods
Bacterial strains, plasmids and growth conditions
The E. coli XL10 Gold (Stratagene, USA) was used as host for plasmids pUC18/19 (6). Cells were grown aerobically at 30°C in a mineral salts medium (8) containing 20 mM D,L-2CP or in Luria-Bertani medium (15). Ampicillin (100 µg/ml) was incorporated as appropriate. Carbon sources and supplements were sterilised separately and added aseptically. Growth was followed by measurement of the absorbance at A680nm. The plasmids used in this study are listed in Table 1.

Plasmids used in this work

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Phenotype</th>
<th>References</th>
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<tbody>
<tr>
<td>pUC18</td>
<td>Amp′, pBR322</td>
<td>(26)</td>
</tr>
<tr>
<td>pFH648</td>
<td>dehE′, dehR′</td>
<td>in this study</td>
</tr>
<tr>
<td>pFH45</td>
<td>dehR′</td>
<td>in this study</td>
</tr>
<tr>
<td>pSC520</td>
<td>dehE′, partial dehR′</td>
<td>(19)</td>
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<tr>
<td>pSC530</td>
<td>dehE′, partial dehR′</td>
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Purification of D,L-2CP
1M solution of D,L-2CP (Aldrich) was acidified by the addition of concentrated nitric acid until the pH was approximately pH 1. The substrate was then extracted three times with ethyl acetate and the inorganic phases were pooled and dried over anhydrous sodium sulphate. The resulting solution was evaporated at 45°C in a rotary evaporator. The residues was then redissolved in distilled water and neutralised with 10M NaOH before being made up to the original volume. This preparation could then be used for growth substrate.

DNA manipulations
Plasmid preparations and DNA ligation were carried out by standard procedures (18). Restriction digests of plasmid DNA were carried out using 0.5-1 µg DNA with 5-10 U restriction enzyme for 1 h at 37°C in the supplied buffers. Restriction fragments were separated by electrophoresis in 0.8% agarose and extracted from gel using a Jetsorb kit (GenomedMED Inc.). Transformations were performed using the Mops/RbCl method (10).

Nucleotide sequencing, oligodeoxyribonucleotide synthesis and computer analysis
Plasmid DNA was prepared using the Wizard Kit (Promega). Sequencing of both DNA strands were carried out using Applied Biosystem-ABI PRISM 377 (BigDye® Terminator v3.0 Cycle Sequencing Kit) automated sequencer. Sequence analysis was carried out using the DNA Strider programme. Sequence alignment using the Genetics Computer Group (GCG) package was used to identify regions of similarity between two sequences.

PCR procedures
Amplification reactions contained in 50 µl: 100 ng template DNA, 25 pmol of each primers forward/reverse, 250 pmol of each dNTP, 20 mM Tris pH 8.8, 10 mM KCl, 10 mM (NH₄)SO₄, 4 mM MgSO₄ and 0.1% (mass/vol.) Triton X-100. After denaturation at 95°C for 5 min, followed by cooling, 1U Vent polymerase was added. The following parameters were used for 30 cycles of: denaturation, 95°C for 1 min; annealing, 52°C for 1 min; extension, 72°C for 2 min. The reaction mixture was electrophoresed on a 0.8% agarose gel.

Chemicals and biochemicals
The chloroalkanoic acids were from Aldrich or Fluka and were purified before use as described (4). Restriction endonucleases, phosphatase and T4 DNA ligase were from Pharmacia. Vent polymerase was from New England Biolabs. All other chemicals were of analytical grade.

Growth of transformants on D,L-2CP
The gene library was transformed into E. coli XL10 Gold supercompetent cells (Stratagene). To improve transformed cell viability, they were first grown in 1.5 ml LB for one hour at 37°C. Then ampicillin (to a final concentration of 100 µg/ml) was added. Following incubation at 37°C overnight the cells were harvested, washed with sterile distilled water, resuspended in sterile distilled water and plated out onto selective D,L-2CP minimal medium plates and incubated at 30°C. Purified D,L-2CP was used in the plates because E. coli K-12 might be inhibited by impurities in non-treated D,L-2CP (3). After 4 days incubation individual colonies that grew were transferred onto a fresh D,L-2CP plate. A single colony (1a) from this plate was then grown on LB/amp for plasmid DNA preparation.

Results and Discussion
The cloning strategy of the putative regulator gene (dehR) together with the dehE gene and construction of a genomic library
It was proposed that dehE and dehR were neighbouring genes (11). The current method of selection will detect the ability of E. coli to grow on a substrate that previously it was unable to grow on. Two main criteria need to be met to achieve this. First, the host needs to be able to take up into the cell the carbon source used for the selection and second the host needs to be able to use the product of dehalogenation as a source of carbon and energy.

A restriction enzyme fragment was identified that would include the whole dehR gene based on the restriction mapping of pSC530 (19). Rhizobium sp. RC1 chromosomal DNA digested with HindIII gave a fragment of 4 kb that hybridized with the Xho1-EcoRI (210 bp) probe. Therefore, the 4 kb HindIII fragment region was extracted from an agarose gel and ligated into pUC18 to produce a gene library.
Choosing a gene probe

Upstream of the dehE gene on the insert DNA of pSC520 was a sequence that was tentatively identified as part of a dehalogenase regulator gene by deduced amino acid sequence comparison with a dehalogenase regulator protein from Pseudomonas putida PP3 (19).

The restriction enzyme sites present in the putative regulator gene sequence that was identified using the DNA Strider programme, indicated that a 210 bp EcoRI and XhoI internal fragment was present. The presence of this fragment was confirmed by restriction enzyme digestion and it was isolated from an agarose gel for use as probe for the dehR gene.

Isolation of putative regulator gene (dehR) together with the dehE gene

Rhizobium sp. RC1 chromosomal DNA digested with HindIII that hybridized with the XhoI-EcoRI (210 bp) probe at the 4 kb DNA fragment was extracted from an agarose gel and ligated into pUC18 to produce a gene library. The plasmid construct was analyzed by restriction enzyme digest that were then confirmed by Southern analysis. Fig. 1a shows DNA agarose gel electrophoresis of plasmid that was digested with appropriate restriction enzymes. Fig. 1b indicates the fragments that hybridised with the EcoRI-XhoI probe. From Fig. 1b it can be seen that a single hybridisation band is present at 3.8 kb in the HindIII digests at Lane 4. There was no hybridisation to the 2.7 kb vector DNA. The plasmid digested with EcoRI (Lane 5) showed the 2.5 kb fragment hybridised with the probe as expected but not the 3.9 kb fragment. In the double digest (Lane 6) with EcoRI and HindIII, the same size (2.5 kb) fragment hybridised but not the 2.7 kb of the vector or the 1.5 kb fragment. In Lane 7 the SalI digest showed the 4.5 kb fragment hybridised. Because there are three SalI sites the larger fragment that hybridised included the vector. The two fragments of approximately 1.4 kb and 200 bp did not hybridise. Fig. 2a shows the deduced restriction map based on the restriction enzyme digests that were confirmed by Southern analysis and the location of the newly cloned putative regulator gene. The plasmid was designated pFH648.

Subcloning of pFH648 was carried out to separate dehE gene from the regulator gene. The first constructed subclone involved the deletion of approximately 2.2 kb to leave a 2.0 kb SalI-HindIII fragment, from map position 2.0 to position 4.0 kb, which carried the putative dehR sequence (Fig. 2a). This pFH648 was digested with SalI and the desired fragment was extracted from the agarose gel. The purified DNA fragment was then re-ligated. The competent E. coli cells were then transformed with the same DNA. Transformed colonies were selected on the basis of their antibiotic resistance. Restriction analysis of the isolated plasmid showed that it contained the expected insert and so the plasmid was designated pH45 (dehR<sup>+</sup>) (Fig. 2b).

Nucleotide sequencing of the putative regulator gene and sequence comparison

The nucleotide sequencing was carried out on the plasmid pH45. The complete dehR gene sequence can be downloaded at Accession Number DQ155290. The initiation codon was ATG (methionine). The reading frame of the Rhizobium sp. dehR gene consisted of 1704 bp, which encoded a 567 amino acid protein with a calculated subunit molecular weight of 63,935 Da.

The amino acid sequence of the Rhizobium sp. putative DehR was also compared to the sequences in the SWISS-PROT-EMBL database. There was high identity with Pseudomonas putida PPF3 regulator protein DehR<i>+</i> (22, 25) that showed 72% sequence identity and 77% similarity when conservative amino acid substitutions were taken into account.

Growth experiment E. coli XL10 Gold::pFH648 (dehE<sup>+</sup>, dehR<sup>+</sup>)

E. coli XL10 Gold::pFH648 (dehR<sup>+</sup>, dehE<sup>+</sup>) was inoculated into minimal medium containing 20 mM D,L-2CP and a control supplied with 10 mM lactate to check if the used source of inoculum was viable. In Fig. 3, growth on D,L-2CP minimal medium can be seen (doubling time 13 hr), which suggests dehE were expressed. IPTG was not added to avoid expression from any genes from the lac promoter system in the plasmid.

**Fig. 1b.** X-Ray film of hybridised fragments

pSC520 hybridised at 4.8 kb-positive control (Lane 1); 1 kb DNA ladder-negative control (Lane 2, 3 and 8); plasmid pFH648 digested with HindIII hybridised at 3.8 kb but not 2.7 kb (Lane 4); plasmid pFH648 digested with EcoRI hybridised at 2.5 kb but not 3.9 kb (Lane 5); plasmid pFH648 digested with EcoRI/HindIII hybridised at 2.5 kb but not at 2.7 kb and 1.3 kb (Lane 6); plasmid pFH648 digested with SalI hybridised at 4.5 kb but not 1.4 kb and 200 bp (Lane 7)

**Fig. 1a.** Fragment analysis of plasmid construct (pFH648) prior to blotting for Southern analysis

Positive control (Lane 1) pSC520 digested with HindIII; 1 kb DNA ladder (Lane 2, 3 and 8); plasmid digested with HindIII (Lane 4); EcoRI (Lane 5); EcoRI/HindIII (Lane 6); SalI (Lane 7)
pUC. *E. coli* without transformed plasmid did not show any growth.

![Growth experiment](image)

**Fig. 3.** Measurement of *E. coli*::pFH648 (*dehR*, *dehE*) growth in 20 mM D,L-2CP minimal medium. Data shown are the means of at least three independent experiments with the error bars indicated.

**Growth experiment *E. coli* XL10 Gold::pSC520 (*dehE*) lacking putative *dehR* gene**

To further investigate the presence of the *dehR* gene sequence involved in dehalogenase gene expression, a clone with partial *dehR* gene was used in the growth experiment. Based on the restriction enzyme mapping of pSC520, the *dehR* gene was 90% deleted on the plasmid construct (19). *E. coli* XL10 Gold::pSC520 (*dehE*) was inoculated into minimal medium containing 20 mM D,L-2CP without IPTG and a control supplied with 10 mM lactate to check if the used source of inoculum was viable (Fig. 4). No growth was observed. Since the cells did not grow in minimal media supplied with 20 mM D,L-2CP as sole source of carbon and energy, it was suggested that a putative *dehR* gene sequence up-stream of *dehE* was needed for *dehE* gene expression to allow growth in D,L-2CP minimal media.

![Gene organization](image)

**Fig. 2a and b.** Restriction map of pFH648 to locate the putative *dehR* gene and *dehE* and subclones to show *dehR* gene in pFH45. Polyclinker region (E-H) not to scale (left hand side). Arrows denote direction of transcription for both *dehE* and *dehR* genes. Hatched area indicates polyclinker region of pUC18 (not to scale). Key to enzymes: H: HindIII; S: SalI; X: XhoI; E: EcoRI.

**Fig. 4.** Measurement of *E. coli*XL10 Gold::pSC520 (partial *dehR*, *dehE*) growth in 20 mM D,L-2CP minimal medium. Data shown are the means of at least three independent experiments with the error bars indicated.

**Growth of *E. coli* XL10 Gold::pFH45 (*dehR*)**

Growth of *E. coli* XL10 Gold::pFH45 (*dehR*) was also tested to see whether growth in D,L-2CP was due to *dehE* gene. *E. coli* XL10 Gold::pFH45 (*dehR*) was inoculated into minimal medium containing 20 mM D,L-2CP without IPTG and a control supplied with 10 mM lactate to check the used source of inoculums if it was viable (Fig. 5). Growth was not observed suggesting that *dehE* is required to allow growth in D,L-2CP. Since both *dehE* and *dehR* were neighbouring genes with opposite direction of transcription, promoters of *dehE* will be predicted by sequence comparison.

**Gene organization of *dehE* and *dehR***

The overall map of pFH648 (*dehE*, *dehR*) showing the relationship between the 2-haloalkanoic acid dehalogenase gene *dehE* and its adjacent regulator gene, *dehR* with opposite direction of transcription. The intergenic region between *dehR* and *dehE* genes (292 bp) from *Rhizobium* sp. was compared to...
the equivalent intergenic sequence from *Pseudomonas putida* PP3 (283 bp) (22). Because of the similar situation to dehR and dehE in *Rhizobium* sp. it might be possible to identify the promoter by sequence comparison. Further analysis need to be carried out to confirm this.

However, dehalogenase enzyme regulation of *Xanthobacter autotrophicus* GJ10 is not well understood (23). The sequence upstream of *dhlB*, the gene encoding haloalkanoic acid dehalogenase, was determined and showed ORFs that may function in transport of acids and regulation of expression of *dhlB*. The protein encoded by the putative dehalogenase regulator, *dhlR*, showed high similarity with proteins from the family of transcriptional activators which activate expression from -24/-12 promoters. Expression from this promoter requires the RNA polymerase factor σ54 and a transcriptional activator. Thus, the expression of the transport protein *dhlC* and possibly *dhlB* may be under the positive regulatory control of *dhlR* dependent on σ54 (23). In *Pseudomonas putida* PP3 the *dehI* was also controlled by -24/-12 promoter as indicated by the lack of expression of *dehI* observed in an *rpoN* mutant of *Pseudomonas putida* PP3 (20). Generally, activation of σ54 dependent promoters occurs in response to a situation of environmental stress (21).

In *Alcaligenes xylosoxidans* ssp. *denitrificans* ABIV there are some indications from restriction patterns and initial sequencing data that a gene encoding a putative σ54-dependent activator, *dhlRm*, similar to the *dehR* regulatory gene from *Pseudomonas putida* PP3 was located upstream of *dhlIV*, a gene encoding haloalkanoic acid dehalogenase. The encoded amino acid sequence of the haloalkanoic acid dehalogenase of *Alcaligenes xylosoxidans* ssp. *denitrificans* ABIV gave 70% identity to *Rhizobium* sp. *DehE* enzyme (19). However, the sequence of *dhlRm* was not available to see the relationship with *Rhizobium* sp. *dehR* gene.

**Conclusions**

The results from the current study presented a strong suggestion that DehR controls expression of the *dehE* gene in *Rhizobium* sp. In *Pseudomonas putida* PP3 the location of *dehR*, is adjacent to *dehI* with the opposite direction of transcription. That is exactly similar to the *dehR* and *dehE* genes in *Rhizobium* sp. Further support for this view was seen in *Alcaligenes xylosoxidans* ssp. *denitrificans* ABIV (2) and *Xanthobacter autotrophicus* GJ10 (23) where positive regulator genes were adjacent to a dehalogenase structural gene but with the opposite direction of transcription. However, in both these cases the only evidence to show that the regulator gene product controlled the structural gene was the presence of the putative σ54 (-12/-24) promoter consensus sequence identified in the upstream region of the structural genes.

Although putative regulator genes have been identified in *Pseudomonas putida* PP3, *Xanthobacter autotrophicus* GJ10 and *Alcaligenes xylosoxidans* ssp. *denitrificans* ABIV, the regulator gene product was not studied further except by sequence comparison. In addition, the sequence homologies in *dehR*, from *Pseudomonas putida* PP3 and the putative promoter sequence are highly speculative.

In future, the physical evidence by gel shift analysis will be carried out based on *in vitro* analysis. A strong interaction of *dehR* gene product with inducer and promoter gene is
expected. Apart from that, genetic analysis can be performed as well to show the actual role of DehR. These findings may provide a novel approach for studying dehalogenase gene regulation system.

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REFERENCES