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## 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 *pFH648* 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*<sup>+</sup>, *dehR*<sup>+</sup>) 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*<sup>+</sup>) lacking *dehR* gene and *E. coli* XL10 Gold::pFH45 (*dehR*<sup>+</sup>) 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*<sub>1</sub> and *dehI*<sub>1</sub>, promoters were predicted to be present for both *dehE* and *dehR* genes.

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**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-dichloropropionic 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**.

**TABLE 1**

Plasmids used in this work

Plasmids	Phenotype	References
pUC18	Amp <sup>r</sup> , pBR322 derived expression vector	(26)
pFH648	<i>dehE</i> <sup>+</sup> , <i>dehR</i> <sup>+</sup>	in this study
pFH45	<i>dehR</i> <sup>+</sup>	in this study
pSC520	<i>dehE</i> <sup>+</sup> , partial <i>dehR</i> <sup>+</sup>	(19)
pSC530	<i>dehE</i> <sup>+</sup> , partial <i>dehR</i> <sup>+</sup>	(19)

### 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 ligations 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% (mass/vol.) 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 (NH<sub>4</sub>) SO<sub>4</sub>, 4 mM MgSO<sub>4</sub> 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 *Hind*III gave a fragment of 4 kb that hybridized with the *Xho*I-*Eco*R1 (210 bp) probe. Therefore, the 4 kb *Hind*III fragment region was extracted from an agarose gel and ligated into pUC18 to produce a gene library.

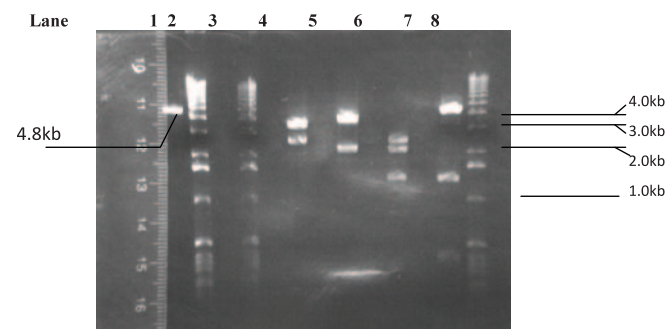
## Choosing a gene probe

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.

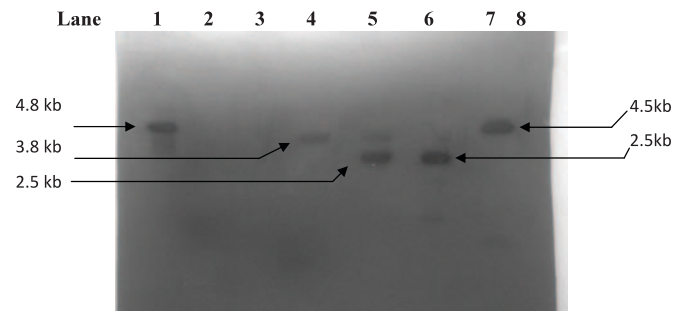
## Isolation of putative regulator gene (*dehR*) together with the *dehE* gene

*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 analysed 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 *Sall* digest showed the 4.5 kb fragment hybridised. Because there are three *Sall* 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.



**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); *Sall* (Lane 7)



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

pSC520 hybridised at 4.8kb-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 *Sall* hybridised at 4.5 kb but not 1.4 kb and 200 bp (Lane 7)

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 *Sall-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 *Sall* 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 pFH45 (*dehR*<sup>+</sup>) (**Fig. 2b**).

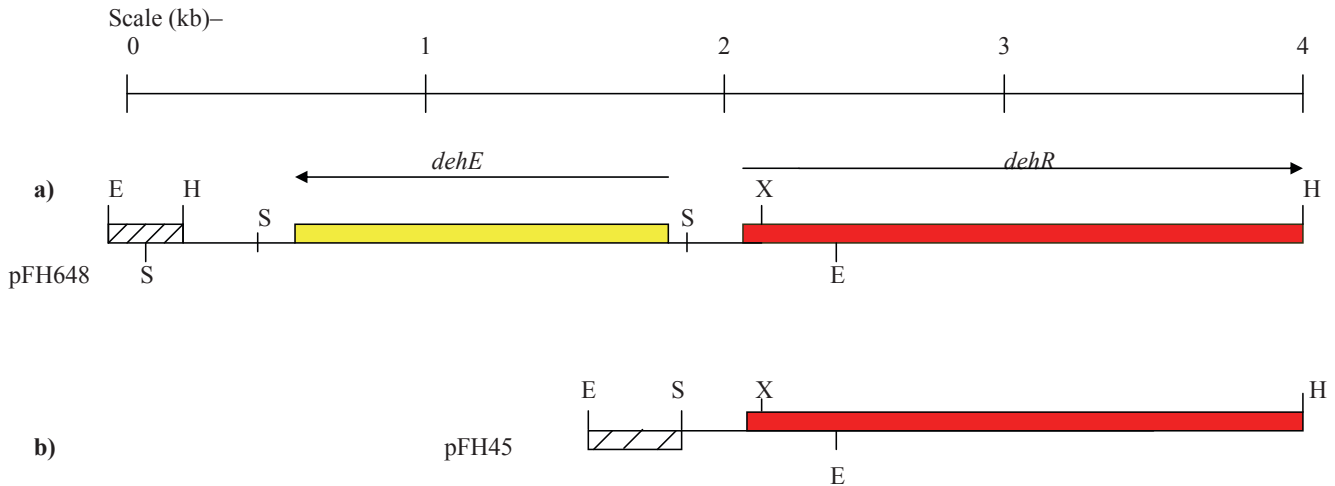
## Nucleotide sequencing of the putative regulator gene and sequence comparison

The nucleotide sequencing was carried out on the plasmid pFH45. 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* PP3 regulator protein DehR<sub>1</sub> (22, 25) that showed 72% sequence identity and 77% similarity when conservative amino acid substitutions were taken into account.

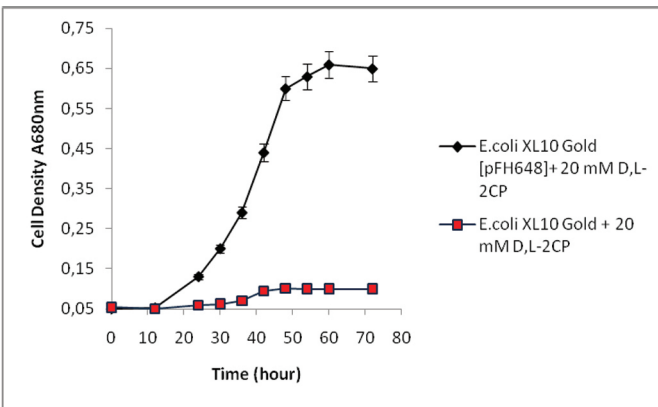
## Growth experiment *E. coli* XL10 Gold::pFH648 (*dehE*<sup>+</sup>, *dehR*<sup>+</sup>)

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

pUC. *E. coli* without transformed plasmid did not show any growth.

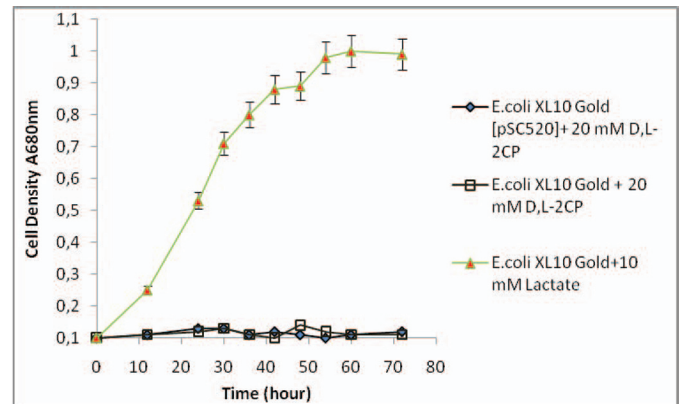


**Fig. 3.** Measurement of *E. coli*::pFH648 (*dehR*<sup>+</sup>, *dehE*<sup>+</sup>) growth in 20 mM D,L-2CP minimal medium

Data shown are the means of at least three independent experiments with the error bars indicated

### Growth experiment *E. coli* XL10 Gold::pSC520 (*dehE*<sup>+</sup>) 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*<sup>+</sup>) 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.



**Fig. 4.** Measurement of *E. coli*XL10 Gold::pSC520 (partial *dehR*<sup>+</sup>, *dehE*<sup>+</sup>) 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

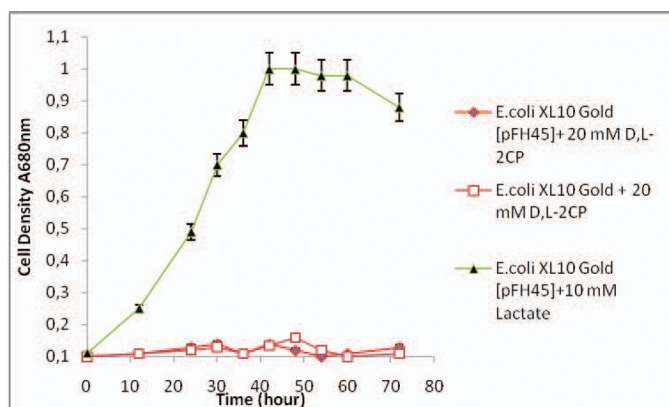
### Growth of *E. coli* XL10 Gold:: pFH45 (*dehR*<sup>+</sup>)

Growth of *E. coli* XL10 Gold::pFH45 (*dehR*<sup>+</sup>) was also tested to see whether growth in D,L-2CP was due to *dehE* gene. *E. coli* XL10 Gold::pFH45 (*dehR*<sup>+</sup>) 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*<sup>+</sup>, *dehR*<sup>+</sup>) 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.



**Fig. 5.** Measurement of *E. coli* XL10 Gold::pFH45(*dehR*<sup>+</sup>) 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

It was proposed that dehalogenase genes in *Rhizobium* sp. were positively regulated with a promoter to control *dehE* and a different promoter controlling the *dehD* and *dehL* genes (11). In the present investigation, the function of DehR in controlling expression of the *dehE* was successfully tested. This was achieved by using a construct carrying structural genes and the regulator gene and monitoring dehalogenase expression in an heterologous host cell by growth on D,L-2CP. The absence of growth suggests that the same promoter and structural gene is needed for the expression of both genes. In this study, it was strongly indicated that expression of *dehE* was triggered by the product of the adjacent gene, *dehR*. The *dehR* transcription was initiated from its own promoter. It was described earlier that based on high similarity of gene sequence organization to the *Pseudomonas putida* PP3 (22, 25), possibly a *Rhizobial* -24/-12 promoter for *dehE* will be present. From the current observation it was suggested that both promoters were recognized by *E. coli* RNA polymerase.

The nucleotide sequence of *dehR*<sub>1</sub> in *Pseudomonas putida* PP3 revealed sequence similarity (in both DNA and deduced protein sequences) to a number of other  $\sigma^{54}$  dependent activator proteins. A putative -24/-12 promoter was identified in the nucleotide sequence upstream of *dehI* by sequence comparison to other consensus' sequence of the -24/-12 promoter. It was predicted that in *Rhizobium* sp. the same putative promoter will be located upstream of *dehE* and possibly required  $\sigma^{54}$ -dependent activator proteins for transcription.

Apart from *dehI* and *dehR*<sub>1</sub> of *Pseudomonas putida* PP3 (22), the genetic organisation of *dehE* and *dehR* of *Rhizobium* sp. with opposite direction of transcription was also identical to 2-haloalkanoic acid dehalogenase *dhlIV* and *dhlR*<sub>IV</sub> of *Alcaligenes xylosoxidans* ssp. *denitrificans* ABIV (2) and *dhlC/dhlB* and *dhlR* of *Xanthobacter autotrophicus* GJ10 (23).

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  $\sigma^{54}$  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  $\sigma^{54}$  (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  $\sigma^{54}$  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  $\sigma^{54}$ -dependent activator, *dhlR*<sub>IV</sub>, similar to the *dehR*<sub>1</sub> 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 *dhlR*<sub>IV</sub> 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*<sub>1</sub> 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  $\sigma^{54}$  (-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*<sub>1</sub> 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

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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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