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TAJUK PROJEK : INVESTIGATION OF RHIZOBIAL DEHALOGENASE CONTROL SYSTEM IN DEHALOGENASE GENE EXPRESSION

Saya FAHRUL ZAMAN HUYOP

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INVESTIGATION OF RHIZOBIAL DEHALOGENASE CONTROL SYSTEM IN DEHALOGENASE GENE EXPRESSION

(KAJIAN SISTEM KAWALAN DEHALOGENASE RHIZOBIAL DALAM PENGEKSPRESAN GEN DEHALOGENASE)

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2009

Investigation of Rhizobial Dehalogenase Control System in Dehalogenase gene expression

(Kajian Sistem Kawalan Dehalogenase Rhizobial dalam pengekspresan gen Dehalogenase)

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Dedication

We would like to thank Ministry of Higher Education (MOHE-Vot 78181) for financial support. We also like to thank Mrs. Fatin Hanani Sulaiman for technical assistance in completing the final report.

Investigation of Rhizobial Dehalogenase Control System in Dehalogenase gene expression

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

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. *Rhizobium* sp. RC1 genetic organization was determined, suggesting *dehE* was controlled by the product of *dehR.* Current study proved previous analysis by growth experiment, *E.coli* XL10 Gold::pFH648 (*dehE*$^+$, *dehR*$^+$) has the ability to grow in minimal media supplied with 20 mM D,L-2CP as sole source of carbon. Whereas, *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 both *dehE* and *dehR* were neighboring genes with opposite direction of transcription, promoters of *dehE*, –24 (TGGCA)/–12 (TTGCTA) and *dehR,* -10 (ACCA)/-35 (AGGT) were identified by sequence homology. Gel shift experiment supported the proposed genetic organisation and regulation for the *Rhizobium* sp. RC1 dehalogenase gene.

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Kajian Sistem Kawalan Dehalogenase Rhizobial dalam pengekspresan gen Dehalogenase

***Kata Kunci**: biodegradation, dehalogenase, bioremediation, dehalogenase regulator, promoter*

Jujukan DNA pada bahagian awalan gen *dehE* mengkodkan enzim dehalogenase E (DehE) *Rhizobium* sp. RC1 telah ditentukan mengandungi frame terbuka yang di panggil *dehR,* yang mengkodkan protein yang mempunyai kesamaan dengan protein dehalogenase regulatory (DehR). DNA plasmid yang di panggil pFH648 yang membawa kedua-dua gen *dehE* dan *dehR* telah di klonkan dari DNA genomic *Rhizobium* sp. RC1. Organisasi genetik *Rhizobium* sp. RC1 telah ditentukan. Ia dicadangkan mengandungi gen *dehE* yang di kawal oleh hasil gen *dehR.* Dalam penyelidkan ini telah membuktikan analisis sebelumnya tentang kewujudan kedua gen *dehE* dan *dehR* melalui ujikaji pertumbuhan bakteria *E.coli* XL10 Gold::pFH648 (*dehE*$^+$, *dehR*$^+$) yang berkebolehan hidup dalam media minimal yang dibekalkan dengan 20 mM D,L-2CP sebagai sumber karbon utama. Manakala, *E.coli* XL10::pSC520 (*dehE*$^+$) tanpa gen *dehR* dan *E.coli* XL10 Gold::pFH45 (*dehR*$^+$) tanpa gen *dehE* tidak tumbuh dalam media minimal yang di bekalkan dengan 20 mM D,L-2CP sebagai sumber karbon dan tenaga utama. Ujian ini mencadangkan bahawa kedua gen *dehE* dan *dehR* amat diperlukan bagi membenarkan pertumbuhan atas media minimal mengandungi D,L-2CP. Seperti yang diketahui dari maklumat lalu, kedua gen *dehE* dan *dehR* adalah berkedudukan bersebelahan antara satu sama lain, dengan arah transkripsi berlawanan masing-masing, dimana promoter *dehE*, –24 (TGGCA)/–12 (TTGCTA) dan *dehR,* -10 (ACCA)/-35 (AGGT) telah di kenalpasti melalui cara penjujukan homolog. Ujkaji "Gel shift" menyokong cadangan organisasi genetik ini dan kawalannya dalam *Rhizobium* sp. RC1 gen dehalogenase.

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CHAPTER 1

1.0 GENERAL 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 (Fetzner, *et al.*, 1994; Janssen, *et al.,* 1994; van Pée and Unversucht, 2003). A soil bacterium isolated by elective culture on DALAPON (2,2-dichloropropionic acid) and identified as a *Rhizobium* sp. was found to produce three haloalkanoate dehalogenases (Leigh *et al.,* 1988). It was curious why this organism produced more than one dehalogenases known as DehD, DehE and DehL.

1.1 General Problem Statements

This study was carried out to understand the control mechanism of dehalogenases in *Rhizobium* sp. RC1, whether a single regulatory gene controls all three dehalogenase gene. For a start, we study how *dehE* was regulated in the presence of the cloned dehalogenase regulator gene. In addition, both genes are neighbouring genes as described in previous study.

1.2 Objectives and scope of research

The objectives of current study is to investigate whether a single regulatory gene (*dehR*) controls *dehE* gene. Both genes were cloned in *E.coli*, and we will demonstrate that whether *E.coli* acquiring both *dehE* and *dehR* able to grow in minimal media supplied with D,L-2CP as sole source of carbon

and energy. Further investigation to detetct the presence of promoters will be carried out by gel shift experiment.

CHAPTER 2

2.0 LITERATURE REVIEW

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 (Fetzner, *et al.*, 1994; Janssen, *et al.,* 1994; van Pée and Unversucht, 2003). A soil bacterium isolated by elective culture on DALAPON (2,2-dichloropropionic acid) and identified as a *Rhizobium* sp. was found to produce three haloalkanoate dehalogenases (Leigh *et al.,* 1988). It was curious why this organism produced more than one dehalogenases when DehE could act on all the substrates that DehL and DehD could act on. *Rhizobium* sp. dehalogenases were studied at a genetic level using a series of mutant strains. 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 positively controlled 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. The mode of regulation of the dehalogenases was proposed from this study by Leigh *et al.,* (1986).To date the proposed relative location of *dehD* and *dehL* genes has been confirmed by sequence analysis (Cairns et al., 1996). *dehD* was located upstream of *dehL* with 177 bp of non-coding region. The third *Rhizobium sp.* dehalogenase *dehE*, was

also sequenced (Stringfellow *et al.*, 1997). 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 part of a putative dehalogenase regulatory gene (*dehR*). The partial of the deduced amino acid sequence showed a significant identity of 51% and similarity of 74% when conservative substitutions were taken into account to the N-terminal region of a *Pseudomonas putida* dehalogenase regulatory gene product (Topping *et al.,* 1995) suggesting that a regulatory gene (*dehR*) was located close to *dehE*. Currently, the regulation of dehalogenase gene expression in dehalogenase producing bacteria is poorly understood. 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* able to grow in minimal media supplied with D,L-2CP as sole source of carbon and energy. Identification of promoters and analysis of regulator gene product supported by the gel shift experiment are presented in this study.

CHAPTER 3

3.0 Materials and methods

3.1 Bacterial strains, plasmids and growth conditions

The *E.coli* XL10 Gold (Stratagene, USA) was used as host for plasmids pUC18 and pUC19 (Gough and Murray 1983). Cells were grown aerobically at 30°C in a mineral salts medium (Hareland *et al.*, 1975) containing 20 mM DL2CP or in Luria-Bertani medium (Miller, 1972). Ampicillin (100 µg/ml) were incorporated as appropriate. Carbon sources and supplements were sterilised separately and added aseptically. Growth was followed by measurement of the absorbance at 680nm.

3.2 DNA manipulations

Plasmid preparations and DNA ligations were carried out by standard procedures (Sambrook *et al.,* 1989). 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 (Kushner, 1978).

3.3 Nucleotide sequencing and oligodeoxyribonucleotide synthesis

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 by employing standard pUC18/19 reverse and forward primers. Sequences were extended by designing downstream primers based on the available determined sequence. These oligonucleotide primers were made with an Applied Biosystem Model 380B synthesizer using cyanoethyl-phosphoramidite chemistry.

3.4 PCR procedures

Amplification reactions contained in 50 µl, 100ng template DNA, 25 pmol each primers forward/reverse, 250 pmol each dNTP, 20 mM Tris pH8.8, 10 mM KCl, 10 mM $(NH_4)SO_4$, 4mM $MgSO_4$ 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 30 cycles of the following parameters were used: 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.

3.5 Chemicals and biochemicals

The chloroalkanic acids were from Aldrich or Fluka and were purified before use as described (Cairns *et al.,* 1996). Restriction endonucleases,

phosphatase and T4 DNA ligase were from Pharmacia. Vent polymerase was from New England Biolabs. All other chemicals were of analytical grade.

3.6 Gel mobility shift assay

DehR protein extracts were prepared from 4 ml of cells in buffer containing 0.1 M Tris acetate reagent, 1 mM EDTA, 10% mass/vol glycerol, pH 7.6. The cells were pelleted by centrifugation at 10 000g for 10 min, 4°C. Cells were lysed by 30 sec treatment at 0°C in an MSE Soniprep 150 ultrasonicator operating at 10μm amplitude and the crude extract was centrifuged at 20 000 g for 15 min at 4°C. Protein concentration was determined using the Bradford assay (Bio-Rad).

An appropriate amount of DehR protein, D,L2-CP and plasmid pSC520 (*dehE*$^+$, *dehE* promoter) digested using *Eco*RI were mixed together at 30°C in the gel shift reactions. Controls without D,L2-CP and DehR protein were prepared at the same time. The reaction mixture was electrophoresed on a 0.8% agarose gel. All gel mobility shift experiments were repeated three times and the data presented are representative example of each experiment.

3.7 Computer analysis

DNA and amino acid sequences were analyzed with the BLAST network service at the National Center for Biotechnology Information (Bethesda, MD). The DNA sequence alignment was constructed using the MultAlin multialignment program (Corpet, 1988).

CHAPTER 4

4.0 Results

4.1 Cloning the putative regulator gene (*dehR*) together with the *dehE* gene and construction of a genomic library

This method of selection will detect a new ability of the host cell 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. Dehalogenation product of D,L-2CP by DehE gives rise to lactate, a normal growth substrate for *E.coli*. A 4kb fragment is needed to include both *dehE* and *dehR* based on the restriction mapping of pSC530 (Stringfellow *et al.*, 1997). Chromosomal DNA was digested with *Hin*dIII (Figure 1.). Chromosomal DNA digested with *Hin*dIII gave a

fragment of 4 kb that hybridized with the *Xho*1-*Eco*R1 (200 bp) probe. Therefore, only the 4 kb *Hin*dIII fragment region was extracted from an agarose gel and ligated into pUC18 to produce a gene library.

4.2 Growth of transformants on D,L-2CP

The gene library was tranformed 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. 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.

4.3 Restriction mapping and Southern analysis

The plasmid prepared from this isolate (1a) was analysed by restriction enzyme digest. Through a series of single and double digests it was possible to confirm the previous map of pSC530 (Stringfellow *et al.,* 1997) and the location of the newly cloned putative regulator gene. Figure 2. shows the deduced restriction map based on the restriction enzyme digests that were then confirmed by Southern analysis. The plasmid was designated pFH648. Subcloning of pFH648 was carried out to separate

dehE gene from the regulator gene. The first subclone constructed involved the deletion of approximately 2.2 kb to leave a 2.0 kb *Sal*I – *Hin*dIII fragment, from map position 2.0 to position 4.0 kb, that carried the putative *dehR* sequence (Figure 3.). For this pFH648 was digested with *Sal*I and the desired fragment was extracted

from the agarose gel. The purified DNA fragment was then re-ligated and transformed into competent *E.coli*. Transformed colonies were selected on the basis of antibiotic resistance. Restriction analysis of the miniprepped plasmid showed that it contained the expected insert and so the plasmid was designated pFH45 (*dehR*$^+$).

4.4 Nucleotide sequencing of the putative regulator gene

The nucleotide sequencing was carried out on the plasmid pFH45. The initial

sequence was obtained by using standard m13 forward and reverse primers. The full sequence of *dehR* gene on both strands was obtained using custom-synthesised oligo deoxyribonucleotide primers. The *dehR* gene sequence can be downloaded at Accession Number DQ155290.

4.5 Growth experiment *E.coli* XL10 Gold::pFH648 (*dehE*$^+$, *dehR*$^+$)

E.coli XL10 Gold::pFH648 (*dehR*$^+$, *dehE*$^+$) was inoculated into minimal medium containing 20 mM D,L-2CP and a control supplied with 20mM lactate to check the source of inoculum used was viable. In Figure 4., growth on D,L-2CP minimal medium can be seen, which suggests *dehE*

were expressed. IPTG was not added to avoid expression from any genes from the *lac* promoter system in the plasmid pUC.

Figure 4. Measurement of *E.coli*::pFH648(*debR*$^{+}$, *debE*$^{+}$) growth in 20 mM D,L-2CP

minimal medium

4.6 Growth experiment *E.coli* XL10 Gold::pSC520 (*dehE*$^+$) lacking *dehR* gene

To further investigate the function of the *dehR* gene to show that it was involved in dehalogenase gene expression a clone with *dehR* largely deleted was used in the growth experiment. Based on the restriction enzyme mapping of pSC520, the *dehR* gene was 90% deleted on the plasmid construct (Stringfellow *et al.,* 1997). *E.coli* XL10 Gold::pSC520 (*dehE*$^+$) was inoculated into minimal medium containing 20 mM D,L-2CP without IPTG and a control supplied with 20mM lactate to check the source of inoculum used was viable. The results showed 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, suggesting promoters and *dehR* regulator gene were needed for *dehE* gene expression to allow growth in D,L-2CP minimal media.

4.7 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 20mM lactate to check the source of inoculum used was viable. Growth was not observed suggesting that *dehE* is needed to allow growth in D,L-2CP. Since both *dehE* and *dehR* were neighboring genes with opposite direction of transcription, promoters of *dehE* will be identified.

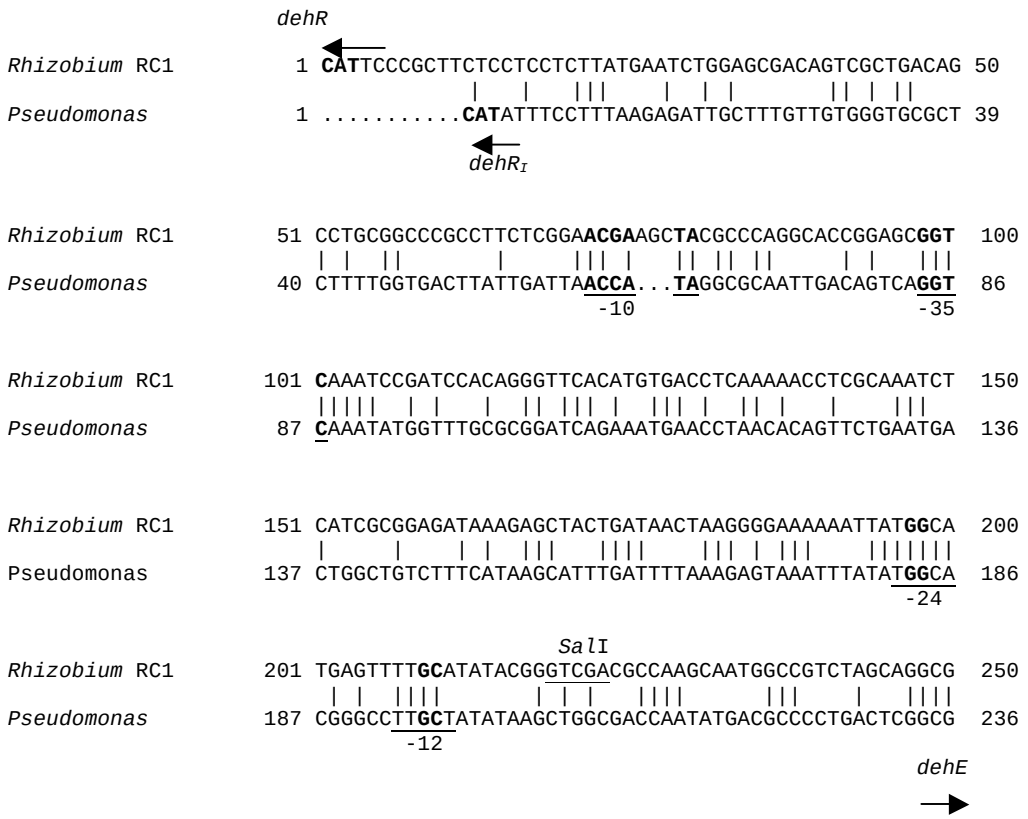
4.8 Identification of *dehE* and *dehR* putative promoters by sequence comparison

In *Pseudomonas putida* PP3 (Topping *et al.*, 1995) upstream of *dehI* was an intergenic region between *dehI* and the regulator gene (*dehR*$_1$) where the promoters of the genes have been tentatively identified. Because of the similar situation to *dehE* and *dehR* in *Rhizobium* sp. it might be possible to identify the promoter by sequence comparison.

The intergenic region between *dehE* and *dehR* genes (292bp) from *Rhizobium* sp. RC1 was compared to the equivalent intergenic sequence from *Pseudomonas putida* PP3 (283bp). The result shows an overall 44% identity. The putative -24/-12 and –35/-10 promoters proposed for *Pseudomonas putida* PP3 were identified in *Rhizobium* sp. RC1, as shown

in Figure 5. These –24/-12 and –35/-10 promoter regions were possibly involved in controlling *dehE* and *dehR* genes, respectively.

The putative promoter regions of the *Pseudomonas putida* PP3 sequence are in bold and underlined. The promoter location of *Rhizobium* sp. is identified on the basis of sequence homology only. The conserved base pairs are indicated in bold at –24(GG) and -12(GC). The *Rhizobium* sp. dehalogenase regulator (*dehR*) and structural gene (*dehE*) start codons are in bold. *Pseudomonas putida* PP3 *dehI* structural gene start codon and $dehR_I$ are also marked in bold.



```
Rhizobium RC1       251 GTTCAATCGCCTCAGTGGATTAGCAA..CGCGTATAGGAGCACAGTCATG  298
                          ||    |        |||  |   |     | |||||||  |   ||
Pseudomonas         237 ATTGTTGCTGACTTACGGACCAAGCATTTATGGATAGGAGAGCCCACACA  286

Rhizobium RC1       299 ...

Pseudomonas         287 ATG
                            dehI
```

Figure 5. Intergenic region sequence comparison between *Rhizobium* sp. RC1 and *Pseudomonas putida* PP3. The putative promoter regions of the *Pseudomonas putida* PP3 sequence are in bold and underlined. The promoter location of *Rhizobium* sp. is identified on the basis of sequence homology only. The conserved base pairs are indicated in bold at –24(GG) and -12(GC). The *Rhizobium* sp. dehalogenase regulator (*dehR*) and structural gene (*dehE*) start codons are in bold. *Pseudomonas putida* PP3 *dehI* structural gene start codon and $dehR_I$ are also marked in bold.

4.9 Gel shift analysis

The possibility of DNA-binding proteins interacting with -24/-12 promoter were

examined using agarose gel with DNA stained with ethidium bromide. DNA binding was observed in protein extracts was increased from 1.0 µg to 1.5 µg (Figure 6.) and affect the gel mobility shift (Figure 6., lane 4,5). Controls did not show any binding and did not affect the gel mobility shift.

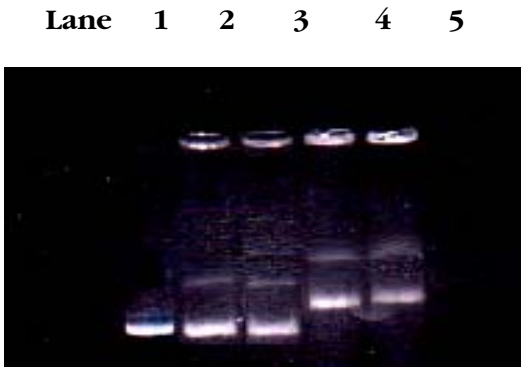


Figure 6. Gel shift analysis of :
Lane 1: pSC520 only
Lane 2 : pSC520 + DehR (1.5 µg)
Lane 3 : PSC520 + D,L-2CP (1mM)
Lane 4 : pSC520 + DehR (1.5 µg) + D,L-2CP (1 mM)
Lane 5 : pSC520 + DehR (1.0 µg) + D,L-2CP (1 mM)

CHAPTER 5

5.0 DISCUSSION

Leigh (1986) 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. 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. To see the functional of both promoters by deletion of promoter sequence was not possible because of the limitation of original restriction enzyme in the gene sequence. It was described earlier that based on sequence homology with the intergenic region of *Pseudomonas putida* PP3 a Rhizobial -24/-12 promoter for *dehE* was identified. From the current observation it was suggested that both promoters were recognized by the by *E.coli* RNA polymerase.

The nucleotide sequence of $dehR_I$ in *Pseudomonas putida* PP3 revealed sequence similarity (in both DNA and deduced protein sequences) to a number of other ^{54} dependent activator proteins. A putative -24/-12 promoter was identified in the nucleotide sequence upstream of *dehI* by sequence comparison to other concensus sequence of the –24/-12 promoter. In *Rhizobium sp.* the same putative promoter was also located upstream of *dehE* and possibly required ^{54}-dependent activator proteins for transcription.

The dehalogenase enzyme regulation of *Xanthobacter autotrophicus* GJ10 is not well understood (van der Ploeg and Janssen, 1995). However, 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 ^{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 ^{54} (van der Ploeg and Janssen, 1995). 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 (Thomas *et al.* 1992). Generally, activation of ^{54} dependent promoters occurs in response to a situation of environmental stress (Thöny and Hennecke, 1989). In *Alcaligenes xylosoxidans* ssp. *denitrificans* ABIV there are some indications from restriction patterns and initial sequencing data that a gene encoding a putative ^{54}-dependent activator, $dhlR_{IV}$, similar to the $dehR_I$ 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 (Stringfellow *et al.*, 1997). However, the sequence of $dhlR_{IV}$ was not available to see the relationship with *Rhizobium sp. dehR* gene.

There was a strong suggestion that DehR controls expression of the *dehE* gene in *Rhizobium sp.* because the two genes were contiguous in the genome. In *Pseudomonas putida* PP3 the location of $dehR_I$ 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 (Brokamp *et al.,* 1997) and *Xanthobacter autotrophicus* GJ10 (van der Ploeg and Janssen, 1995) 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 the regulator gene product controlled the structural gene was the presence of the putative ^{54} (-12/-24) promoter consensus sequence identified in the upstream region of the structural genes (Brokamp *et al.,* 1997; van der Ploeg and Janssen, 1995).

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 further studied except by sequence comparison. In addition, the sequence homologies in $dehR_I$ from *Pseudomonas putida* PP3 and the putative promoter sequence are highly speculative.

In the present study, physical evidence by gel shift analysis was carried out. Based on *in vitro* observation, there was strong interaction of *dehR* gene product with inducer and promoter gene as expected. This finding provides a novel approach for studying protein-DNA interaction in the dehalogenase gene expression system. It was confirmed at the moment that the analysis by gel shift experiment showed a single regulator gene controls expression of *dehE* gene.

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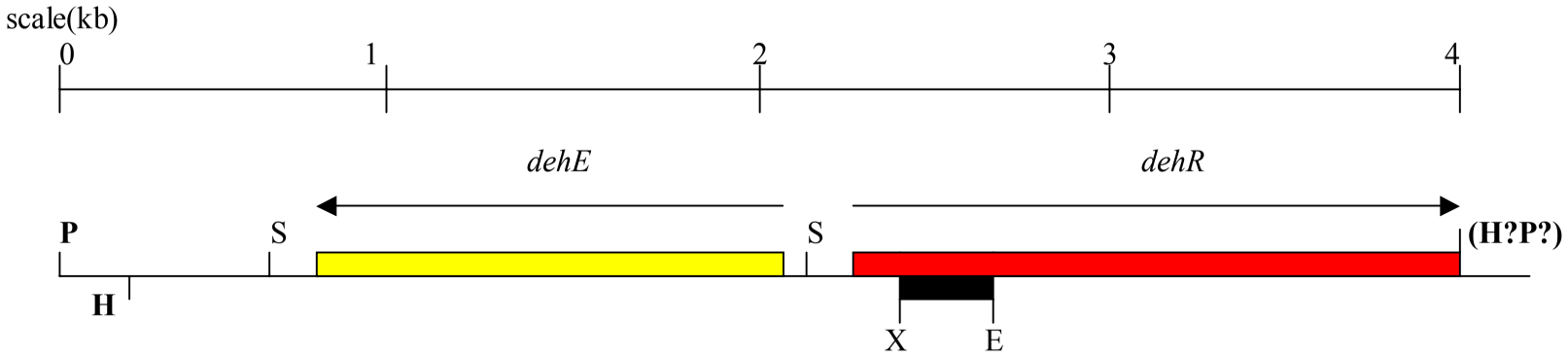

Figure 1. Predicted restriction fragment to include the complete *dehE* and *dehR* genes

*Xho*I and *Eco*RI fragment (solid line) is the 210bp probe. The *dehR* gene is estimated to be about 1.7kb to 2.0kb in length.

(H?, P?) is unknown location of *Hin*dIII and *Pst*I sites in the 3' region of *dehR*. The size of the fragment required to include both genes

is 4kb or above.

Arrows: direction of transcription

Key to enzymes:

P: *Pst*I ; H: *Hin*dIII ; S: *Sal*I ; X: *Xho*I ; E: *Eco*RI

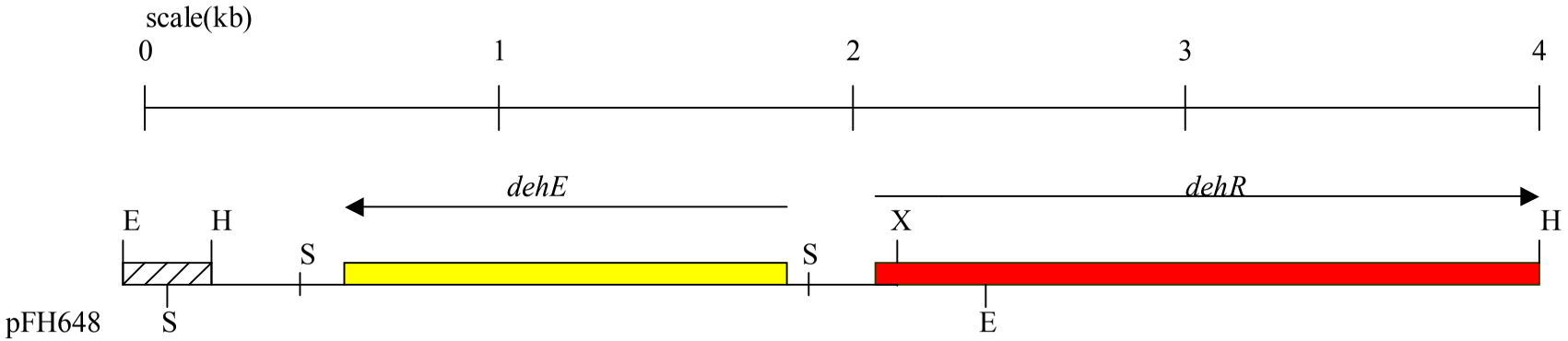

Figure 2. Restriction map of pFH648 to locate the putative *dehR* gene

Polylinker region (E-H) not to scale (left hand side). Arrows denote direction of transcription for both *dehE* and *dehR* genes

Key to enzymes:

H: *Hin*dIII; S: *Sal*I ; X: *Xho*I; E: *Eco*RI

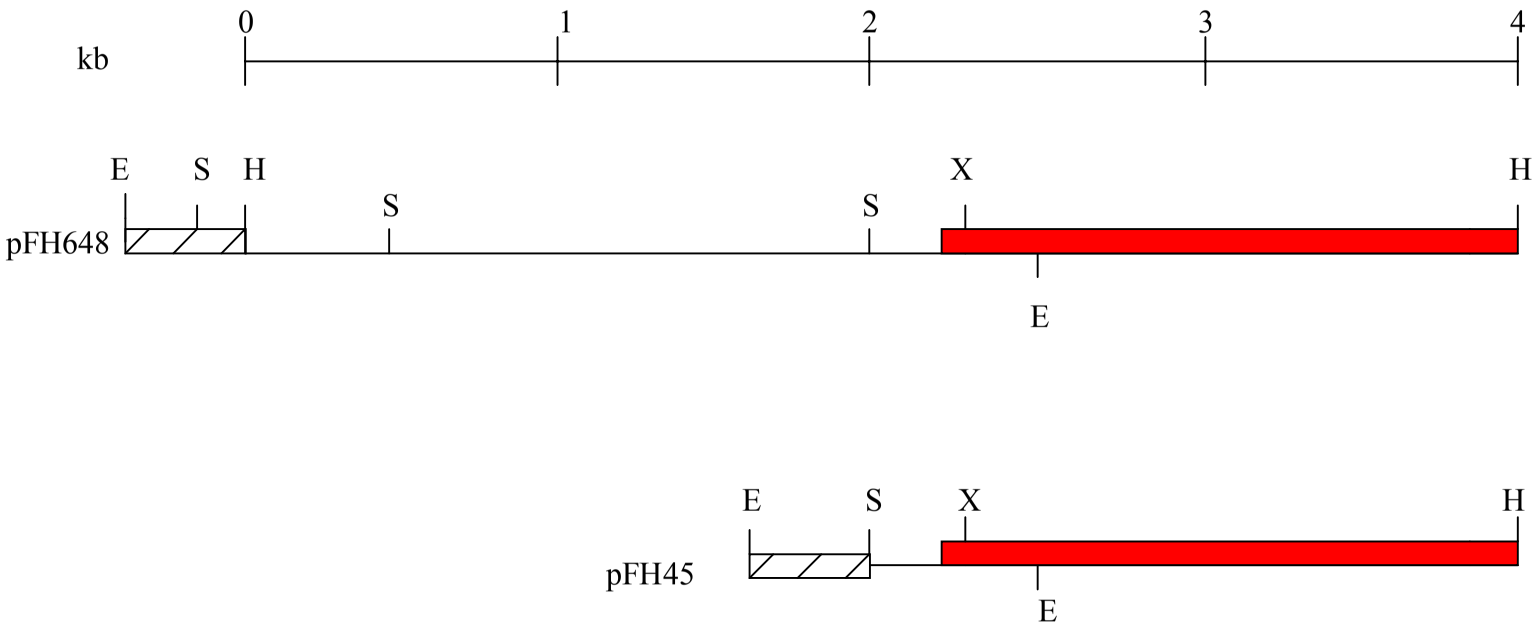

Figure 3. Subclones to show *debR* gene in pFH45

Thick line: *debR* gene

Hatched area: indicates polylinker region of pUC18 (not to scale)

Key to enzymes: