INVESTIGATION OF TRANSCRIPTIONAL REGULATION OF THE HALORESPIRATORY METABOLIC PATHWAY USING AN *IN VIVO* REPORTER ASSAY

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Abstract- The reductive dehalogenase gene clusters in the halorespiratory metabolic pathway are tightly regulated by the transcriptional regulators, CprK and Cprk-like regulators. The presence of the complete genome sequence of *Desulfitobacterium hafniense* strain DCB-2 has made possible the investigation of the transcriptional regulation of the reductive dehalogenase gene cluster, scanning of CprK homologues and identification of putative promoters to examine protein-DNA interaction. The present paper addresses an experimental result of *in vivo* reporter assay which gave insight about transcriptional regulation of the genes encoding the terminal enzyme, reductive dehalogenase that cleave the carbon-halide bond of halocompounds to reduce their toxicity or recalcitrance. The result of *in vivo* reporter study indicated that the putative regulatory genes, cprK2 and cprK4 codes for the transcriptional activators CprK2 and CprK4. The detection of differential transcriptional activation revealed that there are strong and weak promoters that interacted with the transcriptional regulators. 3-chloro-4-hydroxyphenylacetate appears to be a strong effector molecule for the transcriptional regulators, cprK2 and cprK4.

Keywords- CprK2 and CprK4, 3-chloro-4-hydroxyphenylacetate, *Desulfitobacterium hafniense*, Halocompounds, Halorespiratory metabolic pathway.

Introduction
Halogenated Substrates/Halocompounds

Halogenated compounds are class of compounds formed from a hydrocarbon skeleton and a halogen group (Fluorine, Chlorine, Bromine, Iodine). Halogenated compounds are synthesized in nature. Several flora and fauna produce these compounds as natural metabolites. Brominated metabolites are common in marine ecosystem while chlorinated metabolites occur frequently in terrestrial ecosystem. Several halogenated metabolites are synthesized by bacteria, fungi, marine algae, lichens, higher plants, mammals and insects. There existed 1783 organochlorine, 1545 organobromine, 87 organoiodine and 22 organofluorine [12]. Production of chlorine via photolysis of ozone in the presence of sea particles, production of chlorinated compounds in peat bogs and biosynthesis of chlorinated phenols by lily were mentioned. Such organisms as blue green alga, *Oscillatoria agardhii*, the sponge *Theonella* sp., marine sponge, *Discodermia calyx*, red alga *Gracilaria corono pifolia*, Antarctic red alga *plocamium cartilagineum* and fungus *hericium erinaceus* produce halocompounds [12]. The chlorine cycle is a major complex biogeochemical cycles. Halogenated organic compounds have been released in to the environment via anthropogenic, biogenic and geogenic sources. Chlorinated compounds are commonly found in soil, water bodies, insulators, industrial solvents, wood preservatives areas including

many other places. They are produced following destruction of forest by fire. They can be produced by chemical synthesis methods in several ways. Because of acute, chronic toxicity, persistence and bioaccumlation, the presence of organohalides is a major concern and a threat to human and ecosystem health. Polychlorinated organohalides such as PCB and PCE accumulate in anoxic ecosystem, including sediments, soil and ground water. The chemical structures of naturally produced halogenated compounds show a high degree of variability. They may contain single or several halogen atoms. Halogen atoms in organic compounds are important for biological activity. The biological activities of several halogenated compounds rely on the type, number and position of halide ion in the organic compound. Attempts were made to minimize their effect. For instance, one of the well known convention is the Montreal Protocol, which supported the substitution of chlorofluorocarbons present in refrigerators using liquefied propane [28] as cited by [9]. Halocompounds conquer polluted environments and hence they are environmental pollutants. Thus, they endanger the environment when released for they pollute the environment. Halogenated substrates serve as pharmaceuticals (Such antibiotics as chloramphenicol,7-chlorotetracyclin or vancomycin), herbicides, fungicides, insecticides, flame retardants, intermediates in organic synthesis and solvents. Some examples of pesticides are DDT, dicofol, heptachlor, endosulfan, chlordane, aldrin, dieldrin, endrin, mirex, and pentachlorophenol. Chlorophenols are components in bleaching effluents. Their application as herbicide, fungicide and insecticide indicates that these halocompounds may pollute soil and underground water. Also they occur in pulp and paper, and industrial wastewaters [2]. Their occurrence in wood preservatives was reported. During production of chemicals, halogenated compounds are released as by products. Some examples are dioxins, polychlorinated biphenyls and pentachlorophenol. Some types of organochlorides have significant toxicity to plants or animals, including humans. Pentachlorophenols are carcinogenic [3]. 2,4,6-trichlorophenol, 2,3,4,6-tetrachlorophenol, 3,4,6-trichlorocatechol and 4,5,6-trichloroguaiacol are mutagenic. Chlorinated chlorophenols with high number of carbon-chlorine bond could be highly toxic [5]. Some chlorinated compounds may cause low sperm count, testicular cancer and breast cancer for these compounds mimicked the structure of human hormones [18]. Trichloroethene, tetrachloroethene and 1,1,1-trichloroethane pollute soil, groundwater and the atmosphere [8]. 2, 4-dichlorophenol causes increase in liver and spleen weight. 2,4,6-trichlorophenol causes leukaemia and liver cancer in mouse. Pentachlorophenol affects hepatic enzyme [9]. Organochlorine in human plasma could be associated with breast cancer [12]. Generally, they are toxic to plant, animal and human. A good example is DDT. This insecticide affects metabolism as exemplified by calcium metabolism in birds (avifauna). Astonishingly, some chlorinated compounds are safe to human life. They could serve as hormone. For example, 4-chloroindole-3-acetic acid is a chlorinated plant hormone. Several drugs contain chlorine as well. Even some are ingredient of milk of the human female parent. Chloromethanes like chloroform, dichloromethane Chloromethanes like chloroform, dichloromethane, dichloroethene and trichloroethane used as a solvent. Polychlorinated biphenyls are used as electrical insulator. Photodegradation, photochemical degradation and biodegradation are some of the means of de-
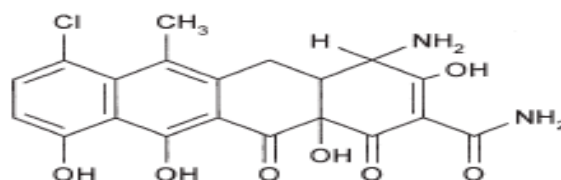
grading halogenated compounds [12]. Biodegradation could be a redox reaction. In this reaction, an electron donor is oxidised while electron acceptor is reduced. Chloromethane is a carbon and energy source for aerobic bacteria, hyphomicrobium and methylobacterium. *Acetobacterium dehalogenans* ferments chloromethane to acetate and Cl- in the presence of carbondioxide. Aerobic and anaerobic bacteria degrade chloromethane via the catalytic role of methyltransferases [7]. Chlorophenols are different in their rate of biodegradability. Among others, the degree of chlorination and the position of the chlorine in the benzene ring matters concerning their relative biodegradability. It is true that they are less biodegradable with high degree of chlorination. The position of the chlorine at ortho, meta and para matters. It was reported that *Desulfitobacterium frappieri* PCP-1 has capabilities of *ortho* dechlorination, meta dechlorination and para dechlorination [26]. However, dehalogenation is not always effective to result intermediates that are less toxic. For example, the meta effect explains the structure and stereochemistry of halogenated compounds affects rate of degradation. For instance, 2,4-dichlorophenoxyaceic acid (2,4-D) is easily degradable when compared to 2,45-trichlorophenoxyaceic acid for the former has chlorine at the ortho position and the latter has chlorine at the meta position [20]. The biodegradability follows the order ortho > meta> para [2].
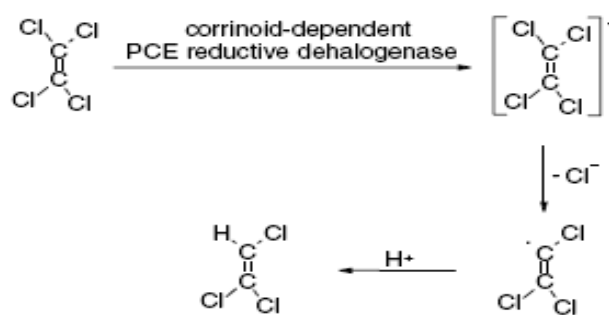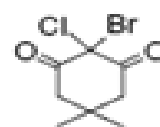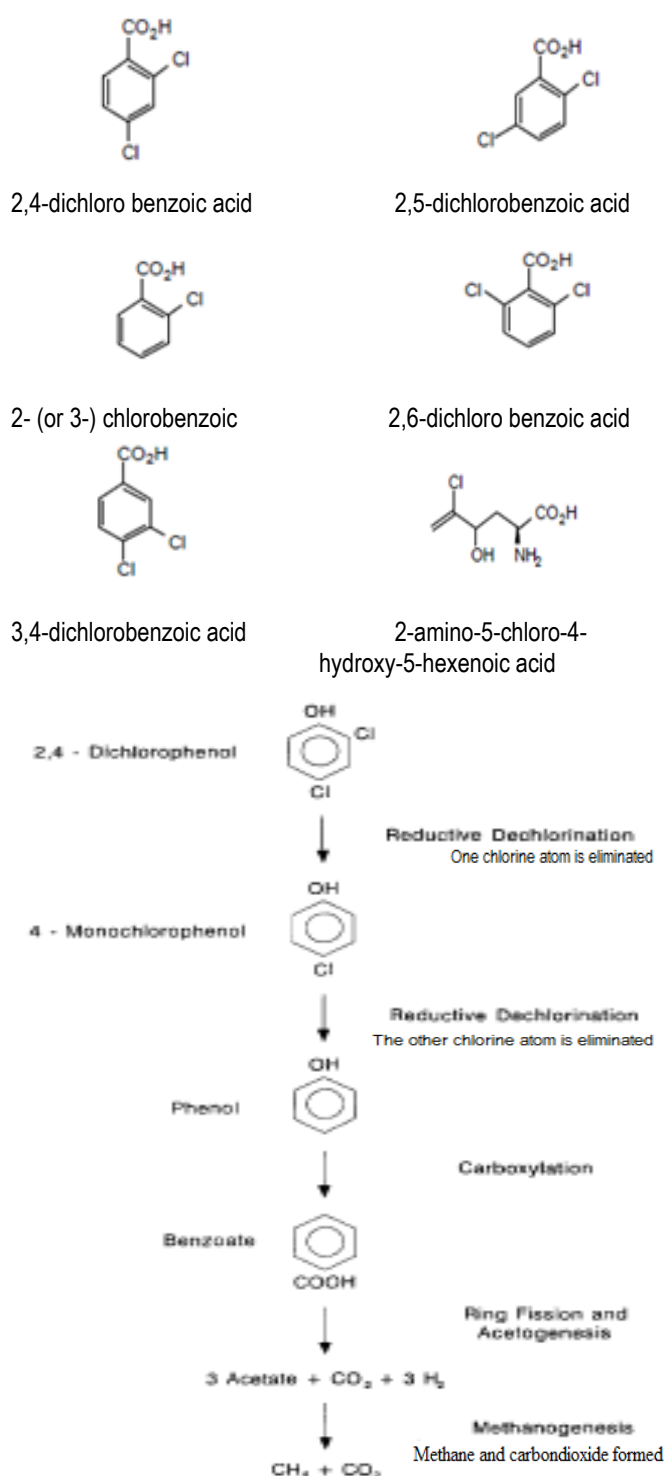

Reductive dehalogenation [24]

(3-chloro-4-hydroxyphenyl acetate) [9]

PCE

4-aminoanhydro-7-chlorotetracycline [25]

(vanPee & Unversucht, 2003a)

2,4-dichloro benzoic acid

2,5-dichlorobenzoic acid

2- (or 3-) chlorobenzoic

2,6-dichloro benzoic acid

3,4-dichlorobenzoic acid

2-amino-5-chloro-4-hydroxy-5-hexenoic acid

Fig. 1- Examples of halogenated compounds, biodegradation and reductive dehalogenation

Carbon-Halide Bond

The carbon-halide bond is formed between carbon and elements of the halogen family. The chlorine is bonded to carbon through covalent bond. This bond is formed between carbon and chlorine, carbon and bromine, carbon and iodine and others. The most frequent bond is the carbon-chlorine bond. The carbon-halide bond is not cleaved by eukaryotes for eukaryotes do not have enzymes that break the bond. The halogen group attached to the hydrocarbon causes toxicity to halogenated compounds. For instance, chlorinated compounds are toxic for a chlorine atom attached to the hydrocarbon skeleton. Removal of the halogen atom from the halogenated compound decreases recalcitrance/toxicity. During breakage of the carbon-halide bond, the halogen substituent is replaced by hydrogen and hydroxyl group [14]. Only few bacteria, which hosted the enzyme reductive dehalogenase can cleave the carbon-halide bond. The process is termed as reductive dehalogenation. The carbon-halide bond occurs in cancer causing halogenated substrates like chlorophenol, 3-chloro-4-hydroxyphenyl acetate, chlorocatechol and others. The toxicity is due to the bond formed between the halogen atom and the carbon. The brominated forms are not as toxic as chlorinated compounds revealing that there is basic difference between the carbon-bromine bond and the carbon-chlorine bond.

There are both anthropogenic and natural productions of halogenated compounds, which contribute to the carbon-halide bond. During breakage of the carbon-halide bond, halogenated compounds serve as terminal electron acceptors in a metabolic pathway, halorespiration (dehalorespiration), which conserves energy for the bacterium. The reaction $R\text{-}Cl + 2\ [H] \circledR RH^+ + Cl^-$ is a typical reduction reaction, which results in replacement of the carbon-chlorine bond by the carbon-hydrogen bond. That means the carbon-chlorine bond is cleaved and replaced by carbon-hydrogen bond during this respiratory pathway. In this case, hydrogen is electron donor while the chlorinated compound is electron acceptor. This reaction is exergonic thermodynamically favored two electron transfer reaction involving release of halogen atom as halide ion. The dechlorination reaction, which removes the chlorine atom is coupled to energy conservation [13]. Halorespiration helps for efficient in situ bioremediation of polluted environment through breaking the carbon-halide bond thereby removing halocompounds [8].

Microbial Dehalogenation

Microbes alleviate accumulation of halogenated compounds for their dehalogenation activities. Dehalogenating microbe has application in practical treatment systems of polluted sites. During microbial dehalogenation, the halogen atom, which is responsible for the toxic and xenobiotic character of halocompound is removed. The removal of the halogen atom decreases recalcitrance and minimize risk of forming toxic intermediates. Generally, microbial consortium dehalogenates and detoxifies organohalides. Anaerobes remediate chlorophenols. However, biodegradation may not necessarily lead to reduced toxic level. For instance, DDT (1,1,1-trichloro-2,2-bis-(p-chlorophenyl) ethane is degraded through the removal of chlorine to result DDE (1,1-dichloro-2,2-bis (p- chlorophenyl) ethylene. DDE is still toxic. Another better example is the degradation of TCE (trichloroethylene). This degradation results in vinyl chloride, which is a potent carcinogen [20]. The genus *Desulfitobacterium*, *Dehalobacter*, *Desulfomonile*, *Desulfuromonas* and *Dehalospirillum* are among the bacterial consortium, which dehalogenated halogenated compounds. It was mentioned that *Dehalococcoides ethenogenes, Desulfomonile tiedjei* DCB-1, *Dehalobacter restrictus* PER-K23, *Dehalospirillum multivorans,Desulfuromonas chloroethenica*, *Desulfovibrio* sp. TBP-

1,and most members of the genus *Desulfitobacterium* are halorespirers [26].
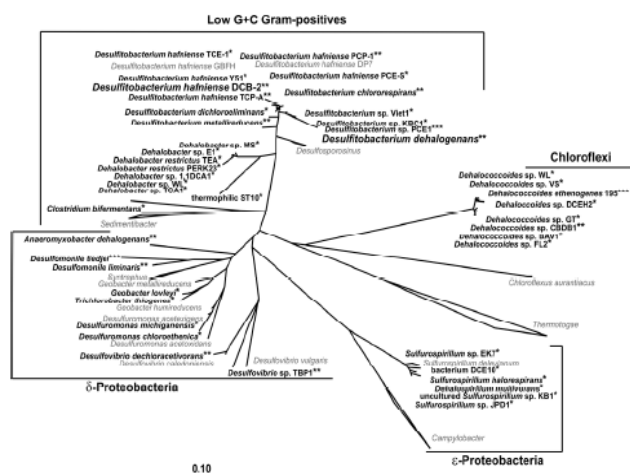

Fig. 2- Phylogeny of Halorespirers

Due to their dehalogenating activities and their respiratory physiology, due attention has been given to the isolation and characterization of these anaerobes that can couple the reductive dehalogenation by specific enzymes to energy conservation via electron transport-coupled phosphorylation in a metabolic pathway, halorespiration/dehalorespiration. In halorespiration, reductive dehalogenation is coupled to energy metabolism. After anaerobic dehalogenation occurs, degradation can be effected with ease aerobically [20]. A halogenated compound, like tetrachloroethene and trichloroethene, serves as a terminal electron acceptor during the oxidation of an electron rich compound, like hydrogen or an organic substrate, which are used as electron donor. To date, the genome sequence of various organisms is released and sequencing projects is increasing at alarming rate. This will pave an opportunity to characterize genes, proteins and promoters using the sequence data obtained from the genome sequence to expand research in microbial dehalogenation. Now, the complete genome sequence of *Desulfitobacterium hafniense* is already available in 2000 at the JGI website. Formerly, the partial genome sequence of *Desulfitobacterium dehalogenans* was available. This has simplified the study of dehalogenation process through tools of molecular biology. At earlier times, bacteria capable of growing under halogenated compounds were isolated and characterized. These microbial consortiums seem to have enormous potential for in situ bioremediation of polluted anoxic environments. *Desulfitobacterium hafniense is* anaerobic dehalogenating bacteria. It dechlorinates aromatic and alkyl chlorinated compounds. It also dehalogenate such problematic pollutants as chlorinated phenols, chlorinated ethenes. It was suggested that it may dechlorinate polychlorinated biphenyls. Some halorespirers belong to the gram positive, low G+C content and spore-forming bacteria. *Desulfitobacterium hafniense* strain DCB-2 undergo chlororespiration. It uses pyruvate, lactate and formate as electron donor. Besides halogenated compounds, it uses elemental sulfur, sulfite, fumarate, nitrate, As (V) and Fe (III) as electron acceptor. The knowledge of microbial dehalogenation will have immense applications in in-situ bioremediation. However, translating remediation effort in to ground will be hindered by a number of factors. Generally, such factors as chance of the introduced microbe to be eaten by predators, failure of the microbe to have access to the halogenated substrate and inability of the microbe to outcompete native microbiota will affect efficiency of in situ bioremediation [20].

Dehalobox

Dehalobox is a regulatory region of bacterial DNA, which regulates transcription of the reductive dehalogenase genes via interacting with transcription factors. Bacteria have promoter at -10 region, TATA box (Pribnow box) represented by the consensus sequence, TATAAT. Also they have promoter at -35, upstream region, which is represented by the consensus sequence, "TTGACA". Dehalobox is a promoter DNA discovered in the genus *Desulfitobacterium*. Dehalobox is located at -41.5 upstream of the transcription start site, +1. The consensus sequence of dehalobox is TTAAT-N4-ATTAA. The first and the last five nucleotides are highly conserved while the middle four are not conserved. Currently, the complete genome sequence of *Desulfitobacterium hafniense* has made possible scanning of several putative dehaloboxes, which needs to be tested experimentally. Dehalobox is palindrome, an inverted repeat containing 14 nucleotides. Formerly, other boxes like CRP box, TGTGA-N_6 - TCACA and FNR box, TTGAT-N_4 - ATCAA were known to science.

Regulator-Dehalobox (Protein-DNA Interaction)

Three regulators, namely, CprK1, *CprK2* and *CprK4* found to interact with dehalobox through their helix-turn-helix (HTH) motif. The recognition helix of the HTH motif binds to dehalobox through molecular interaction between nucleotides from dehalobox and amino acids from HTH motif. The amino acids, which interact with dehalobox are V—SR in case of *CprK1* and *CprK2* while in case of *CprK4*, the amino acids are V—SK [10]. Dehalobox - regulator interaction is studied using techniques of *in vivo* promoter probe assays/ in vivo reporter assay. Transcriptional activation through the interaction between putative regulators and dehalobox" was investigated using this technique. The plasmids pAK80 and pET24d propagated in the *E. coli* strain DH5α were used for an in vivo reporter system. In pAK80, a PCR amplified promoter was cloned while in a pET24d, a regulator was cloned and overexpressed in *E. coli* JM109(DE3),cloning host.
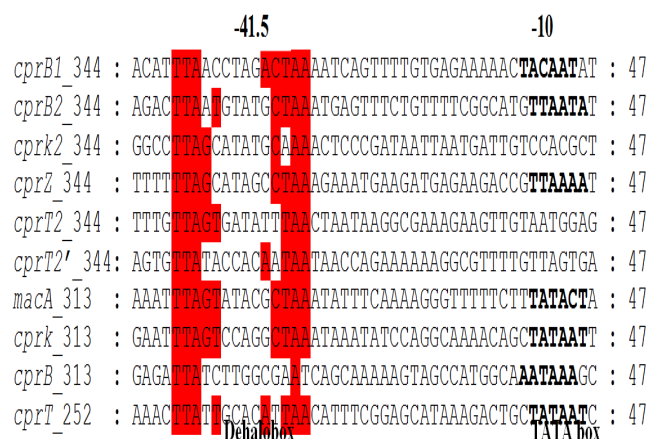

Fig. 3- Multiple sequence alignment of dehalobox

The regulator was found to interact with the promoter with differential rate of transcription [10]. The alignment of dehalobox is shown in Fig 3. The numbers 344, 313 and 252 indicate contig. The letters like B, K, T indicate genes. For example cprB_313 means that that promoter or dehalobox is upstream of the gene cprB.

Transcriptional Regulation Of Reductive DehalogenaseGenes
Mechanisms of gene expression in halorespiring bacteria were investigated in *D. dehalogenans* for the first time. Following addition of halogenated substrate, it was proved that transcription of the reductive dehalogenase encoding cprBA operon was induced 15 fold within 30 minutes. Such evidences of gene expression analysis in the gene cluster cprTKZEBACD, isolation and characterization of mutant incapable of halorespiration revealed that the halorespiratory metabolic pathway is controlled by regulators and environmental sensors. Chaperonins like CprD, CprE and trigger factor CprT are relevant for maturation of reductive dehalogenase. CprK might have turned on the halorespiration gene cluster. Also dehaloboxes, were identified. It was hypothesized that dehalobox might be involved in CprK binding. The genome of *D. hafniense* contains at least 17 Crp-Fnr homologues, five of which are homologues to *D.dehalogen* CprK. CprK bound to cpr promoter only in the presence Cl-OHPA but not the dehalogenated product, OHPA. The gene cprC was found to be expressed during halorespiration in *D.dehalogenanse*. cprC codes for a protein with significant similarity to putative membrane bound transcriptional regulators of NirI/NosR-family [22].

It becomes clear that the reductive dehalogenase gene is regulated at transcription level by five *Cprk-like* regulators, *cprK1, cprK2, cprK3, cprK4* and *cprK5* in *Desulfitobacterium hafniense*. Paralogues of CprK activated transcription from dehalobox [10]. Formerly, *cprK* was found to be transcriptional activator in *Desulfitobacterium dehalogenans* [19]. One of the regulator, *CprK4* activated transcription from a gene that codes methyl-accepting chemotaxis protein revealing that *Desulfitobacterium hafniense* sense halogenated aromates and exhibit a bacteria motility, chemotaxis towards chloroaromate [10]. The *CprK* genes are redundant. In the physical region of cprK genes, a potential transposase-encoding gene was found and perhaps the redundancy of cprk genes could be caused by mobile genetic elements (transposons). CprK1 proved to activate transcription of chlorophenol reductive dehalogenase genes and its effector is 3-chloro-4-hydroxyphenylacetate [10]. Other effectors of CprK1 are 2,3-dichlorophenol, 2,4-dichlorophenol, 2-bromo-4-chlorophenol, 2,4,5-trichlorophenol and 2,4,6-trichlorophenol [17]. The newly identified transcriptional regulator of reductive dehalogenase genes, that is, CprK is already a member of Crp-Fnr transcription regulators family. Presently, the CprK homologues CprK-like regulators will be a new member of Crp-Fnr family. Cprk is a Crp-Fnr homolog involved in regulation of halorespiration. Reductive dehalogenase required for halorespiration are regulated by CprK protein via transcription regulation of the cpr genes which are clustered in cprTKZEBACD. CprK interacts with conserved motifs at the promoter DNA of cpr genes. The mechanism of regulation involves ligand binding to the regulator, which brings conformational change that able the regulator to interact with nucleotide sequence. For instance, CprK protein interacts with the promoter of

CprB gene at a recognition motif TTGAATacgcACTAA positioned at-41.5. The existence of several recognition motifs for CprK-like regulators and different ligands (chlorphenols) have been reported.

The Crp-Fnr family, which contained CprK and CprK like regulators is a major class of transcription factors encompassing a number of proteins. The amino acids in the transcription factor interact with base pairs in promoter DNA via hydrogen bond, ionic bond or van der waals force. The family was under intensive study since the birth of pioneer members, Crp (cyclic adenosine monophosphate receptor protein) and Fnr (a fumarate and nitrate reductase regulator). The family members of these transcriptional regulators increased to 369 proteins following the rapidly increasing microbial genome sequence [16]. These transcriptional factors give explanation for mechanisms of regulation of metabolic pathways in various microbial lineages. Regulation of metabolic pathway allows bacteria to respond to various biological clocks. The family member shares common structural and functional attributes. Generally, the N-terminus domain posses a nucleotide-binding domain like the cAMP-binding protein and their C-terminal domain has a helix-turn-helix motif that interacts with promoter DNA. This motif contained two alpha helices and a short turn between the helices. It interacts with promoter of inverted repeat. Regulator having HTH motif are dimers. Many Crp-Fnr regulators function as transcription activators and repressors in rare cases. Signal molecules are involved in transcriptional regulation during regulator-promoter DNA interaction. Signal molecules can directly interact with Crp-Fnr regulators and bring modification in the conformation of the regulator thereby activating the regulator. A prosthetic group like iron-sulfur group or heme, designed for the interaction with oxygen, NO or CO is another means of interaction. Phylogenetic analysis revealed that ArcR, CooA, CprK, Crp, Dnr, FixK, Flp, Fnr, FnrN, MalR, NnrR, NtcA, PrfA, and YeiL are the major subfamilies. This family is named using the two proteins Crp and Fnr. Crp is a signal molecule that binds to a conserved sequence motif, TGTGA-N_6–TCACA, in the promoter of target genes. Crp regulates such biological processes as the metabolism of sugars or amino acids, transport processes, protein folding and toxin production or pilus synthesis. Fnr is a very close homologue of Crp. It is FeS protein. Fnr from *E.coli* is an oxygen responsive transcription regulator. Fnr binds to Fnr box/anaerobox, (TTGAT –N4-ATCAA). Fnr regulates anaerobic respiratory functions. Fnr is analogous to the catabolite gene activator protein (CAP), which controls catabolite-sensitive gene transcription.

Fig. 4- Multiple sequence alignment

(CprK and other members of CRP-FNR transcriptional regulator superfamily) [21]. In the recognition helix, Valine, Serine and Lysine/Arginine are conserved. Glycine is conserve in the turn structure.
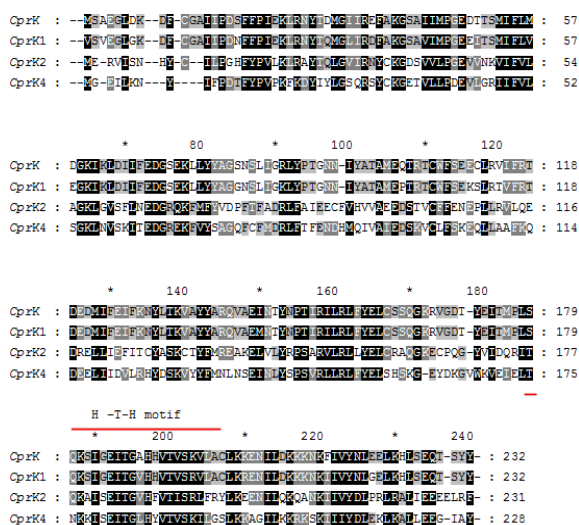

Fig. 5- Multiple sequence alignment of CprK-like regulators

In the recognition helix, Valine, Serine and Lysine/Arginine are conserved. Glycine is conserve in the turn structure.
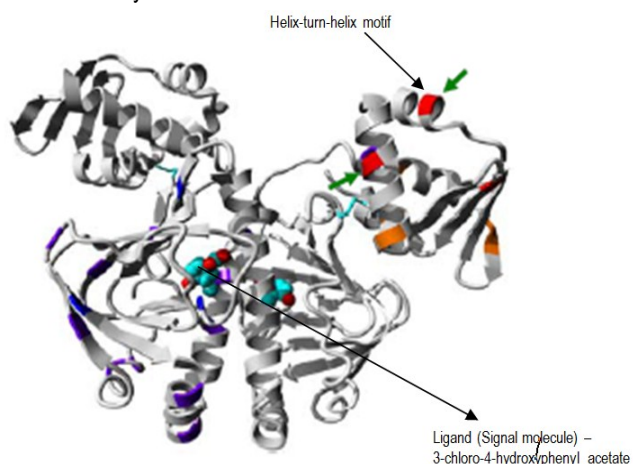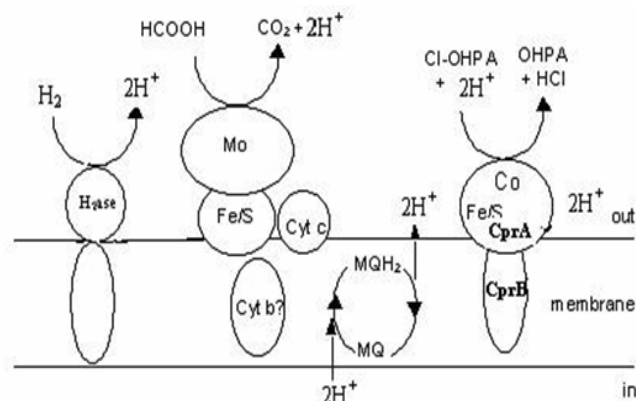

Fig. 6- Crystal structure of *CprK1* [17]

Fig. 7- Halorespiration, Halorespiratory Metabolic Pathway.

H_2ase = hydrogenase Cyt = Cythochrome MQ = Menaquione. When hydrogen losses electron, it becomes H^+ and the electron released will pass through the various electron carriers like Cyt, MQ and FeS. Finally, that electron will be used to reduce the terminal electron acceptor, toxic ClOHPA to non toxic OHPA. During the process, proton is pumped to the outside and the proton motive force generated is utilized to synthesize ATP to be utilized by the bacterium [22]
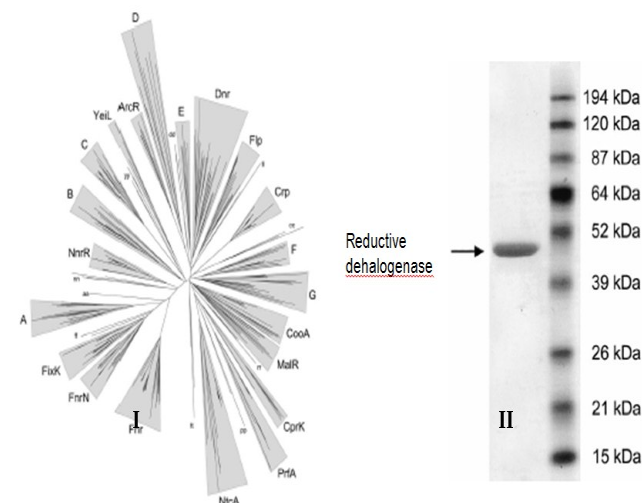

Fig. 8- I. Phylogeny of Crp-FNR transcriptional regulators [16]. II. 3-chloro-4-hydroxyphenylacetate (Cl-OHPA) reductive dehalogenase purified from *Desulfitobacterium hafniense* [4]

Reductive Dehalogenase Genes

Bacteria have genes connected to one another unlike eukaryotes which have a single gene. A group of genes organized together are clusters. Genes in gene cluster are co-transcribed to polycistronic mRNA. The translated product encoded from these genes could be polypeptides or full length proteins. These set of genes may be regulated by a single promoter or divergent promoters with similar regulator sequences. Between each gene, there is a stop codon. A stop codon between mRNA of different gene allows the synthesis of different proteins. At the transcription start site, there is a ribosome binding site, the shine-dalgarno sequence, represented by the consensus sequence, 5'AGGA 3'. Bacterial structural genes that encode for a single metabolic pathway are contiguous. This arrangement is called operon. Operon structure is a feature of bacterial genomes. Operons are transcribed in to a single mRNA molecule. Co-transcribed genes may encode proteins of the same metabolic pathway. Genes tend to switch on and switch off. There exist protein-binding regulatory sequences in genes. During binding to regulatory sequences of a gene, transcriptional activation or repression may occur. Bacterial cells are highly programmed via adjusting alterations in the nutritional environment to regulate cell growth and cell division. For example, the lac operon has promoter and operator, which control mRNA synthesis. Transcriptional regulation by a repressor and activator is negative regulation and positive regulation respectively. The chlorphenol reductase operon, that is, the reductive dehalogenase gene cluster is annotated and characterized well [10]. The regulatory network is available. The genome of *D. hafniense* encodes five CprK-like regulators. Several CprK binding

motifs are in place [10]. Generally, the protein CprK is a transcriptional activator of the halorespiration gene cluster [19]. Currently, CprK1, CprK2 and CprK4 are proved to be transcriptional activator. The reductive dehalogenase genes are hosted in the halerespiring bacteria. 25 phyla of prokaryotes exist and this is entirely constituted from archaea and bacteria [20]. Reductive dehalogenase genes are hosted in the genome of *Desulfitobacterium, Dehalobacter, Desulfomonile, Desulforomonas, Desulfovibrio, sulfurospirillum, trichlorobacter, proteobacterium including enterobacter, clostridium* and *dehalococcoides* [6]. As aforementioned, the first halorespirer known to science is *Desulfomonile tiedjei* [27]. It dechlorinates 3-chlorobenzoate. So far most of the isolates are from *Desulfitobacterium spp.* [1]. There are versatile species degrading chlorinated ethenes or chlorophenols [21]. 2,4,6-trichlorophenol and 3-chloro-4-hydroxy-phenyl acetate are reduced by *Desulfitobacterium hafniense* [13]. Reductive dehalogenase is a novel enzyme that degrades halogenated compounds. It is a terminal enzyme in the halorespiratory pathway. The biocatalyst ortho-chlorophenol reductive dehalogenase from D.dehalogenans is also available [23]. 3-chloro-4-hydroxyphenyl acetate reductive dehalogenase genes occur in cluster as an operon. The nucleotide sequence of the *cprBA* genes encode the catalytic subunit and putative membrane anchor of the *o*-chlorophenol reductive dehalogenase in *D. dehalogenans*. The reductive dehalogenase genes were identified through northern blot analysis of the reductive dehalogenase genes.

There are six transcribed genes, namely, *cprC, cprD, cprE, cprK, cprT*, and *cprZ*, and one untranscribed open reading frame. All genes but *cprT* are transcribed in the same direction. *cprT* gene is monocistronic. *cprBA* and *cprZE* genes are biscistronic. Occasional read-through at *cprC* gives rise to a tetracistronic *cprBACD* transcript. Transcription of *cprBA* was induced 15-fold upon addition of the *o*-chlorophenolic substrate 3-chloro-4-hydroxyphenylacetic acid within 30 min with concomitant induction of dehalogenation activity. Putative regulatory protein binding motifs that to some extent resemble the FNR box were identified in the *cprT-cprK* and *cprK-cprZ* intergenic regions and the promoter at *cprB*, suggesting a role for FNR-like CprK in the control of expression of the *cprTKZEBACD* genes. Reductive dehalogenases consists of eight genes with the order *cprTKZEBACD* and with the same polarity except for *cprT*. The deduced *cprC* and *cprK* gene products belong to the NirI/NosR and CRP-FNR families of transcription regulatory proteins, respectively. CprD and CprE are predicted to be molecular chaperones of the GroEL type, whereas *cprT* may encode a homologue of the trigger factor folding catalysts. *cprK* encode a polypeptide of 233 residues. There is significant homology between CprK and the CRP-FNR regulator family. Almost all FNR-like proteins posses a H-T-H motif at the C-terminus, and E—SR is conserved in their recognition helix, which binds to promoter DNA. From multiple sequence alignment made between CprK and representative family members, the recognition helix has a conserved glutamic acid and serine and arginine (E-SR). Cprk has only Serine conserved at the recognition helix of CprK suggesting that CprK motif may bind to a different sequence other than the FNR box Tte GAT-N_4-ATCAA that binds the FNR-like proteins. The gene *cprK* is constitutively expressed at a low level, encoding a potential transcription regulatory protein. The observed tight control of the expression of the structural and putative accessory *cpr* genes might imply a direct involvement of CprK in the functionality of the *D. dehalogenans* halorespirational system. CprK has significant similarity to FNR- and FixK-like regulators, which are important *trans*-acting factors in regulatory networks of anaerobic assimilation and dissimilation. Like FixK, CprK lacks the N-terminal cysteine cluster, a characteristic of FNR, which is involved in the binding of an Fe/S center, and as such in redox sensing. However, CprK contained five cysteine residues. The internal cysteine residue Cys^{105} is conserved. Northern blot analysis revealed that expression from putative promoters preceding *cprT* and *cprZ* was activated under halorespiring conditions [21]. The characterization of putative dehalogenases and determining their catalytic strategy will be rational to design dehalogenases that can degrade toxic chlorinated compounds.
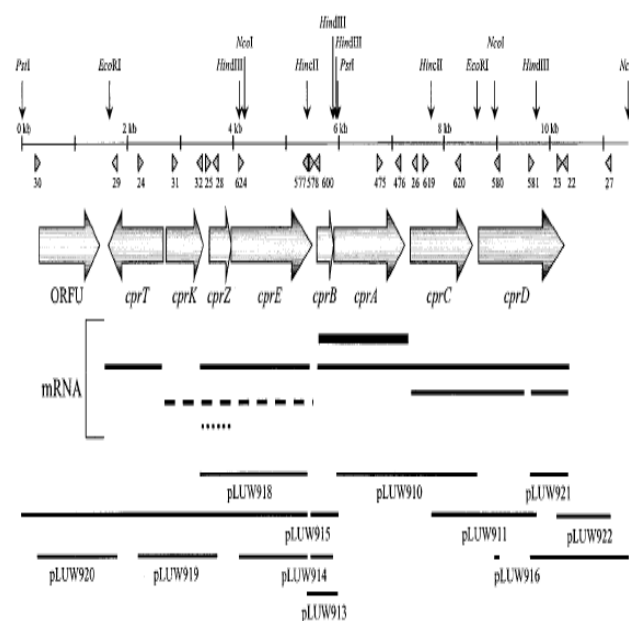

Fig. 9- Cpr gene cluster [21]

Reductive Dehalogenase

There is large variety in dehalogenating enzymes and mechanisms for the degradation of halogenated compounds. Such dehalogenase families as haloalkane dehalogenases, haloacid dehalogenases, 4-chlorobenzoate-CoA dehalogenases break the carbon-halogen bond through substitution mechanisms. The reaction is mediated through a covalent aspartyl intermediate [15]. These enzymes and halorespirers were identified via for their capability to cleave halogenated compounds. Trichloroethene reductive dehalogenase from Dehalococcoides were isolated [14]. Several bacterial isolates were found to couple reductive dehalogenation to energy conservation by electron transport coupled phosphorylation. This is implemented in a metabolic pathway, halorespiration or dehalorespiration. It has been documented these chlorinated aromatic and aliphatic compounds are known to be terminal electron acceptors in an anaerobic reductive dehalogenation metabolic process named halorespiration. Also there are alternative electron acceptors like nitrate and sulfate [11]. The reductive dehalogenases isolated from several bacteria such as *Dehalococcoides ethenogenes* or *Desulfitobacterium dehalogen-*

ans catalyzing these reactions contain a cobalamin cofactor and iron–sulfur clusters. The reductive dehalogenase CprA contains a mixed [4Fe-4S] cluster protein, carrying in each 48 kda monomer a cobalamin molecule in addition to the Fes cluster [23]. The membrane-bound 3-chlorobenzoate dechlorinating enzyme from the gram-negative, sulfate-reducing D*esulfomonile tiedjei* was the first reported enzyme to science regarding terminal enzymes of the halorespiratory pathway. The membrane-bound 3-chloro-4-hydroxyphenylacetate (Cl-OHPA) reductive dehalogenase from the chlorophenol reducing anaerobe *Desulfitobacterium hafniense* was purified. The purified enzyme were found to convert Cl-OHPA to 4-hydroxyphenylacetate in experiments of in vitro catalysis [4]. Dehydrohalogenation, Hydrolytic dehalogenation, Thiolytic dehalogenation, Intramolecular substitution, Oxidative dehalogenation, Reductive dehalogenation, Dehalogenation by hydration,Dehalogenation by methyltransfer and anaerobic reductive dehalogenation (halorespiration) are some of the mechanisms of dehalogenation [25]. All enzymes characterized are tightly associated with cytoplasmic membrane. The function of the cprB gene products has been predicted to be membrane anchor for the biocatalyst. All catalytic RD subunits are synthesized as immature proteins, which consists of a twin Arginine signal sequence. The two arginine signal translocates the complex to cytoplasmic membrane. Also the existence of twin arginine signal revealed that reductive dehalogenases are extra-cytoplasmic enzymes. Reductive dehalogenases have a molecular weight of 50–65 kDa. They contain a cobalamin cofactor and iron–sulphur clusters.

Material and Methods

The study was conducted to examine protein-DNA interaction (Dehalobox-regulator/CprK2 & CprK4 interaction). Polymerase chain reaction, restriction analysis in agarose gel and in vivo assay were some of the techniques used. The PCR program was 90oC 7 min, 90 oC 30 sec, 48 oC 30 sec, 72 oC, 120 sec and 72oC, 7 min for amplifying the regulator gene. The promoters were amplified using 90oC 7 min, 90 oC 30 sec, 46 oC 30 sec, 72 oC, 48 sec and 72 oC, 7 min. Pfu turbo DNA polymerase was used for its high fidelity. Transcriptional regulation of halorespiration was studied using techniques of *in vivo* reporter assay. As shown in Fig 10, pET24d is a vector in which the regulator (CprK2 and CprK4) is cloned and in pAK80 the promoter, putative dehalobox is cloned. To clone the regulatory gene, the forward primer when designed has HindIII site and the reverse primer has NcoI site. To clone the promoter, in the forward primer, BamHI site was incorporated while in the reverse primer has Hind III site was incorporated. pAK80 is a low copy number shuttle vector, which replicate in both gram positive and gram negative bacteria. It has p15A and PCT1138 replicon, shine dalgarno sequence, coding sequence of β-galactosidase without promoter, polylinker and erythromycin resistant gene. pET24d has PBR322 origin, f1 origin, lacI coding sequence, T7 promoter, kanamycin resistant gene and polylinker. The insertion of the promoter in pAK80, that is, the promoter probe construct was checked via selection of DH5α strains in erythromycin containing media. The success of the cloning of the regulatory gene was accomplished by selecting DH5α strains in kanamycin containing media. Ligation of the vector and the DNA was effected by T4 DNA ligase. DNA sequencing was conducted to sequence the cloned promoters in pAK80 and the regulatory

gene in pET24d to confirm the success of the cloning. From pET24d, the regulators CprK2/CprK4 are overexpressed. For this the T7 promoter system along with T7 RNA polymerase of *E.coli* strain JM109 (DE3) was used. Transformant was selected in LB media containing kanamycin and erythromycin. IPTG is added for induction and the ligand for CprK2/CprK4, that is, 3-chloro-4-hydroxyphenyl acetate, was also added. In pAK80, the promoter is cloned near to β-galactosidase coding sequence. Once the regulator is overexpressed, it will bind to the promoter and the enzyme, β-galactosidase will be synthesized after translation of its coding sequence. This reporter enzyme, β-galactosidase will cleave the colorless substrate, ONPG (o-nitrophenyl-b-D-galactoside) to galactose and nitrophenol, which is yellow in color. Since nitrophenol has absorption at 420 nm, the promoter activities was measured by quantifying nitrophenol through measurement using spectrophotometer. b-galactosidase activity was measured in Miller units following computing via Miller units equals to 1000 x specific activity (A420/incubation time x volume x OD600). In the target experiment 0.1mM IPTG & 20mM ClOHPA were added. There were three controls. In control-1, IPTG was added, but ClOHPA was not added. In control 2, no IPTG was added, but ClOHPA was added. In control 3, both IPTG & ClOHPA were added, but pET24d was empty vector meaning that it has no cloned regulator. From control 1, the relevance of the ligand for the conformation of the regulator was checked. From control 2, it was possible to check leakage of transcription without IPTG. Since no regulator is in control 3, it is possible to know if there is transcription or not in the absence of the regulator. The sample of cell culture was harvested at 0 hour during the first induction and in one hour difference since the time of induction. Overnight culture was also taken. The β-galactosidase activity data generated from the spectrophotometer was analyzed using excel spread sheet and SPSS.
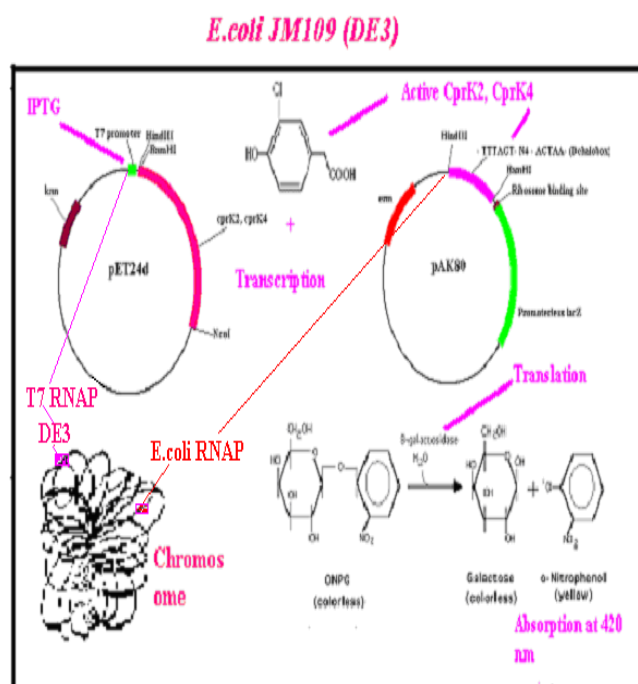

Fig. 10- *In vivo* Reporter Assay.

Results
PCR and Electrophoresis
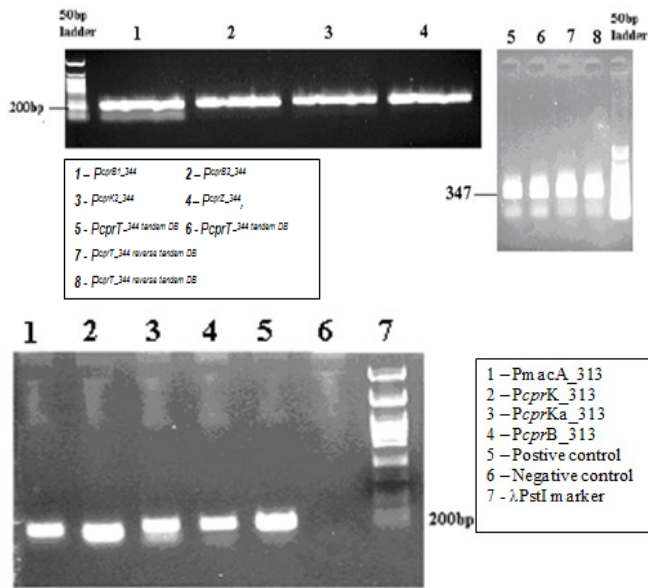The results of the DNA work are shown in Fig 11, 12, 13 and 14.

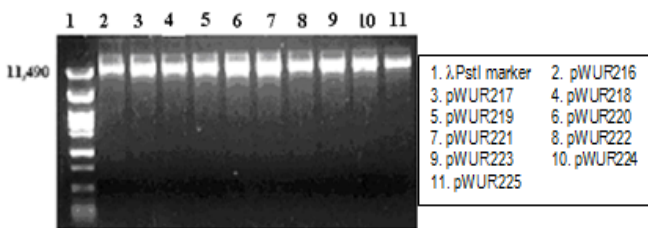**Fig. 11-** PCR product of the promoter DNA, dehalobox

Fig. 12- The promoters in the plasmid pAK80 (a cloned promoter)
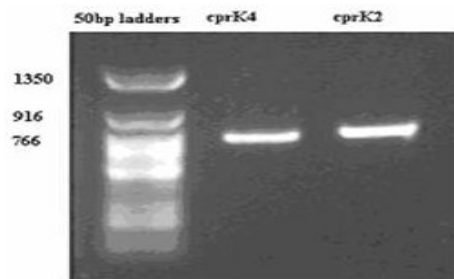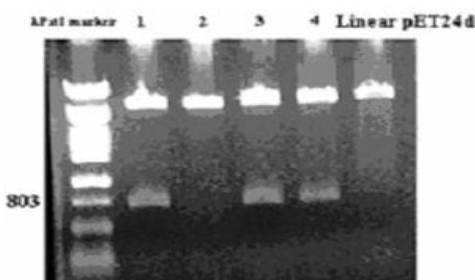

Fig. 13- PCR product of *cprk4* and *cprk2*

Fig. 14- Restriction analysis of *cprk2* and c*prK4 (The digested product cprk2* and c*prK4 and the plasmid are separated after digestion)*

Enzyme (B-Galactosidase) Activity Measurement
The results of the part of the work related to enzymes and protein are shown below.

Table 1- β-galactosidase activity

Promoter (Dehalobox)	B-galactosidase activity (Miller units)	Promoter DNA sequence
PcprB1_344	137.23	T-TTAACCTAGACTAA-A
Control 1	25.34	
Control 2	0.68	
Control 3	6.59	
PcprK2_344	131.4	C-TTAGCATATGCAAA-A
Control 1	15.8	
Control 2	0.69	
Control 3	6.38	
PcprB2_344	51.78	C-TTAATGTATGCTAA-A
Control 1	17.11	
Control 2	0.45	
Control 3	3.99	
PcprZ_344	35.15	T-TTAGCATAGCCTAA-A
Control 1	13.5	
Control 2	0.61	
Control 3	0.81	
PcprT_344 tandem DB	27.09	G-TTAGTGATATTTAA-C
Control 1	8.99	
Control 2	0.39	
Control 3	4.16	
PcprT_344 reverse tandem DB	10.1	G-TTATACCACAATAA-T
Control 1	4.99	
Control 2	0.66	
Control 3	0.62	

In the following pictures, results obtained from the various promoter activities are shown. The promoter in the upstream region of the gene is designated as P and the gene is designated as cpr. The number like 344 indicated contig. In all of the experiments, the first bar shows the highest value since the experiment has both the inducer, IPTG and the ligand, ClOHPA.
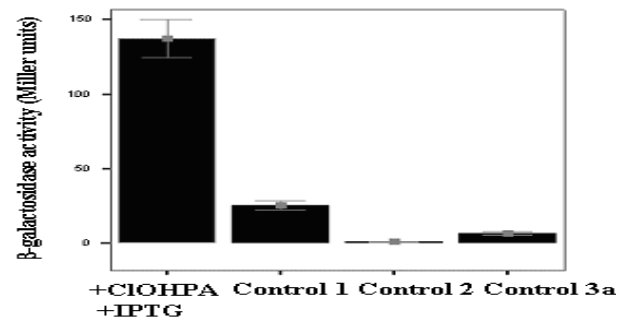

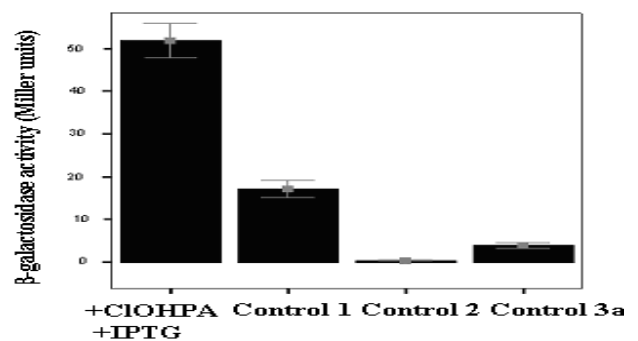**Fig. 15-** *In vivo* activity of P*cpr*B1_344

Fig. 16- *In vivo* activity of P*cpr*B2_344

Fig. 17- *In vivo* activity of P*cprK*2_344

Fig. 18- *In vivo* activity of promoter *cpr*T_344 tandem DB

Fig. 19- In vivo activity of PcprZ_344

Fig. 20- *In vivo* activity of P*cpr*T_344 reverse tandem DB

Fig. 21- *In vivo* activity of P*cpr*T_252

Fig. 22- Comparison of activities of the different promoters

Protein Sequence Analysis

The amino acid sequence of CprK was used as a query sequence in BLASTP analysis. Those homologues to CprK in the genus Desulfitobacterium was taken for further analysis. And the BLASTP result of selected species is shown in Table 2. The alignment of the amino acid sequence revealed that Valine, Serine and Lysine or Arginine are conserved in the recognition helix of these regulators (Fig 23).

Fig. 23- Amino acid sequence alignment

Out of the homologues, one (CprK) from the genome of *Desulfitobacterium dehalogenans.* Eight of the homologues are from *Desulfitobacterium hafniense.* One homolog belongs to *Desulfitobacterium* chlororespirans while the last one homolog existed in *Desulfitobacterium autotrophicum.* Most of the homologs were found in *D. hafniense.* This could be due to the availability of the complete genome sequence of this bacterium. As mentioned, previously five CprK-like regulators were reported. These additional homologs were not reported. Interestingly, the three additional homologs may also attract experimentalists to test these putative regulators. Obviously, all of them belong to Crp/Fnr family tran-

scriptional regulator. The phylogeny analysis of CprK revealed that there are eight Cprk paralogues in *D. hafniense* while there are one ortholog in *Desulfitobacterium dehalogenans*, *Desulfitobacterium autotrophicum* and *Desulfitobacterium* chlororespirans. Events of gene redundancy is detected (Fig. 24).

Table 2. List of homolog of CprK in the genus *Desulfitobacterium* based on BLASTP analysis

| S. No	Accession number	Species	Max score	Total score	Query average	E-value	Description
1.	2H6C_A	*Desulfitobacterium dehalogenans*	481	481	100%	4e-134	putative transcription regulator
2.	2H6B_A	*D.hafniense*	441	441	100%	5e-122	
3.	YP_002457233	*D.hafniense*	439	439	100%	1e-121	cyclic nucleotide-binding protein
4.	3E5U_C	*D.hafniense*	437	437	100%	5e-121	
5.	AAL87760	*D.hafniense*	384	384	87%	6e-105	putative transcription regulatory protein Crp/Fnr family transcriptional regulator
6.	YP_002457187	*D.hafniense*	183	183	92%	1e-44	putative transcription regulatory protein CRP/FNR family transcriptional regulator
7.	YP_002457209	*D.hafniense*	179	179	93%	4e-43	transcriptional regulator, familyCrp/Fnr
8.	YP_002459080	*D.hafniense*	167	167	94%	1e-39	transcriptional regulator, Crp/Fnr family
9.	YP_002457214	*D.hafniense*	163	163	95%	2e-38	putative transcription regulator, Crp/Fnr family
10.	AAG43484	*D.chlororespirans*	159	159	95%	3e-37	CRP/FNR family transcriptional regulator
11.	YP_002603668	*D.autotrophicum*	73.6	73.6	85%	2e-11	transcriptional regulator, Crp/Fnr family
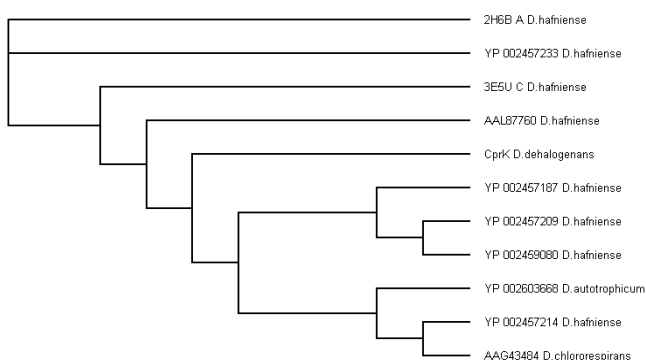
Fig. 24- Phylogeny of Cprk in the genus Desulfitobacterium.

Discussion

It was possible to quantify transcription rate as shown in the aforementioned graphs and tables. The variation in the nucleotide sequence of the dehalobox affected rate of transcription. Also the type of the gene in the gene cluster matters. The highest promoter activity was measured for the promoter located upstream of the operon, cprB₁A. This is convincing for this operon codes for the biocatalyst, reductive dehalogenase. A higher activity (131 Miller units) was observed for the promoter upstream of p^{CprK2}. Since this promoter is upstream of the reguator, CprK2, it seems that the regulator itself is autoregulated. Controls were self explanatory. In the absence of ClOHPA, there was a promoter activity. This reveals that the halorespiration gene cluster is not fully switched off in the absence of this chloroaromate. Perhaps CprK2 in the absence of the ligand bind to the promoter with minimal affinity. All in all it was possible to prove that CprK2 is a transcriptional activator. Attempts to zoom in to the genomics of this bacterium remains to be the sole option to envisage all the homologues and find out their role as transcriptional activator via testing the putative regulatory genes. It seems that both protein and nucleotide sequence analysis are quite fundamental. Conserved residues at the recognition helix seems to be relevant for understanding protein-DNA interaction. Scanning homologues will have a profound impact to document candidate genes. The overall result revealed that CprK2 and CprK4 are transcriptional activators since the first experiment shown in the first bar in all experiments showed a higher activity relative to the controls. Perhaps the detection of higher activity in control 1 (no ClOHPA and IPTG) could be due to the binding of the regulator to the promoter with minimal affinity since the ligand is important for improved interaction. The sequence of dehalobox, the palindrome seems crucial. For instance, the promoter upstream of CprK2 showed high activity. This promoter seems perfectly palindromic. Indeed it seems evident that the middle unconserved nucleotided are also relevant.

Conclusion

In conclusion, microbial dehalogenation is a preferable approach instead of breaking the carbon-halide bond by chemical method. The isolation and characterization of halorespiring microbes seems to be a significant step. Improving the catalytic efficiency of reductive dehalogenase by site targeted mutagenesis is also a novel approach to maximize the catalytic efficiency of the enzyme. Mining and characterizing reductive dehalogenase genes and regulatory regions through tools of bioinformatics is the way to go. The present in vivo study gave insight on how dehalobox promoter is interacting with Cprk-like regulators. Since, this in vivo study is conducted in *E.coli* under aerobic condition, in vivo experiment using the anaerobe *Desulfitobacterium hafniense* will be the strategy to avail the complete understanding of the regulatory network of halorespiration. Engineering *Desulfitobacterium hafniense* through transforming the bacterium with an efficient biocatalyst, reductive dehalogenase and a strong promoter that will induce the catalyst is highly recommended. Attempting in situ bioremediation of polluted environment with maximum care using the genetically engineered *Desulfitobacterium hafniense or Desulfitobacterium dehalogenans* is suggested. The chemistry of the signal molecules or the ligands remains to be the governing factor for the regulator and the biocatalyst. Thus, stereochemistry of aliphatic and aromatic compounds is worth studying. The mechanism of reductive dehalogenation and electron carriers in the electron transport chain must be envisaged in a detailed manner though a lot is already known. It appears that research must be conducted

to test the interaction of CprK2 with such other signal molecules as 2,4,5-TCP, 2,4,6-TCP, 2,4-DCP and 3,5-DCP besides ClOHPA. Nucleotide substitution in the middle uncoserved four bases of dehalobox by mutating the nucleotides is crucial to compare the strength of dehalobox towards having a complete insight in the regulatory network of the halorespiratory metabolic pathway. Since in the present study, the promoters tested against the regulator in the same contig, it is recommendable that one promoter from one contig and regulator from the other contig must be envisaged to have insight on diverse ways of interaction. The crystal structure of CprK1 is already resolved. But, to increase our knowledge about three dimensional structure of the regulator, crystallizing the other regulators like CprK1, CprK2, CprK3, CprK4 and CprK5 will be of paramount importance. The presence of crystal structures will be of considerable significance to investigate mechanism of ligand binding to a regulator, understand the conformation of regulator along with the helix-turn-helix motif including and the kinetics of the enzyme including the catalytic mechanism and strategy. Ultimately, truncating the regulator via amino acid substitution at the recognition helix of the regulator will give clue on how bending of DNA and conformation of the regulator occurs at optimal condition. Approaches of in silico biology to address the bioinformatics of both the biocatalysts encoding gene and the regulatory gene will be a forefront issue.

Acknowledgements

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