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BIDIRECTIONAL GENE SEQUENCES WITH SIMILAR HOMOLOGY TO FUNCTIONAL PROTEINS OF ALKANE DEGRADING BACTERIUM *PSEUDOMONAS FREDRIKSBERGENSIS* DNA

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Abstract

The potential for two overlapping fragments of DNA from a clone of newly isolated alkanes degrading bacterium *Pseudomonas frederiksbergensis* encoding sequences with similar homology to two parts of functional proteins is described. One strand contains a sequence with high homology to alkanes monooxygenase (alkB), a member of the alkanes hydroxylase family, and the other strand contains a sequence with some homology to alcohol dehydrogenase gene (alkJ). Overlapping of the genes on opposite strands has been reported in eukaryotic species, and is now reported in a bacterial species. The sequence comparisons and ORF_S results revealed that the regulation and the genes organization involved in alkane oxidation represented in *Pseudomonas frederiksberghensis* varies among the different known alkane degrading bacteria. The *alk* gene cluster containing homologues to the known alkane monooxygenase (*alk*B), and rubredoxin (*alk*G) are oriented in the same direction, whereas alcohol dehydrogenase (*alk*J) is oriented in the opposite direction. Such genomes encode messages on both strands of the DNA, or in an overlapping but different reading frames, of the same strand of DNA. The possibility of creating novel genes from pre-existing sequences, known as overprinting, which is a widespread phenomenon in small viruses. Here, the origin and evolution of the gene overlap to bacteriophages belonging to the family Microviridae have been investigated. Such a phenomenon is most widely described in extremely small genomes such as those of viruses or small plasmids, yet here is a unique phenomenon.

Introduction

The discovery of overlapping genes, i.e., DNA sequences simultaneously encoding two or more proteins in different reading frames, has exerted a fascination on evolutionary biologists. Such phenomena are relatively common in DNA and RNA viruses (Keese & Gibbs, 1992; Pavesi, 2006). Overlapping genes (OGs) are defined as adjacent genes whose coding sequences overlap partially or entirely. In fact, they are ubiquitous in microbial genomes and more conserved between species than non-overlapping genes (Cheng *et al*., 2010). However, few reports of overlapping genes have been found in mammalian genomes (Tomohir *et al*., 2007). For example, two genes being encoding on one strands are illustrated by a 70 kDa heat shock protein and another NAD-specific glutamate dehydrogenase being found on opposite strands of the DNA in the freshwater mould *Achlya klebsiana* (Walker, 1999). One DNA sequences can code for more than one gene product by using different reading frames or different initiation codons. Several overlapping genes are found in bacterial and eukaryotic genomes, and

are relatively rare in non-viral organisms and few reports have been described overlapping genes in mammalian genomes (Sander & Schulz, 1997; Dan *et al*., 2002; Pagan & Holmes, 2010). Some studies have demonstrated that the overlapping of genes differs among species and have inferred that this can be attributed to differences in evolutionary histories (Veeramachaneni *et al*., 2004; Torresi, 2002). Adachi & Lieber (2002) reported that some genes overlap in a head-to-head manner (transcribed in opposite directions), while Koyanagi (2005) reported the occurrence of bidirectional gene pairs in some species. In a similar manner, a long, antiparallel, coupled open reading frame (LAC ORF) has been described in the antisense strand of a 70K heat shock gene from *Drosophila auraria* (Konstantopoulou *et al*., 1995) and such gene has been described on the antisense strand in *Schizosaccharomyces pombe* (Usui, 1997; Rancure *et al*., 2009). In prokaryotes, Silke (1997) reported a long antisense open reading frame (ORF) on the antisense strand of the *dna*K homologue of *E. coli* but does not indicate the frequency of this sequence in other bacterial species, nor does he assign a function to this ORF. It was suggested that it is one of the few *hsp70*s which contains ORFs.

This work identifies the presence of antiparallel, coupled open reading frame (*alk*B and *alk*J ORFs) sequences with homology to alkane monooxygenase and alcohol dehydrogenase genes lying on the complementary strand of a DNA sequence encoding alkane hydroxylase. The implications of this study are discussed from the perspective of the genes subcloning.

Materials and Methods

Growth of *Pseudomonas (P) frederiksbergensis* and isolation of DNA: *P. frederiksbergensis* was isolated from in earlier work of Abdel Megeed & Mueller (2009). Isolated DNA from *P. frederiksbergensis* was dissolved in TE buffer and stored at –20°C for further experiments according to Pither *et al*., (1989).

PCR amplification of DNA and cloning of PCR product: The amplification was employed for isolation of the gene probe for alkane hyroxylase cloning with the primers Forward: 5`-TGGCCGGCTACTCCGATGATCGGAATCTGG-3` and Reverse: 5`-CGCGTGGTGATCCGAGTGCCGCTGAAGGTG-3 (Innis *et al*., 1990). PCR was performed using 30 cycles of DNA amplification were performed using a melt temperature of 95°C for 60 s, an annealing temperature of 72°C for 5 min., and an amplification temperature of 72°C for 5 min. The nucleotide sequences obtained were entered for BLAST searching (DNA databases) into the web site of the National Center for Biotechnology Information (Altschul *et al*., 1997). All buffers and enzymes were from Fermentas, Roche, NEB and Pomega. The PCR products were cloned in the pUc19 vector, transformed into *E. coli* DH5α and expressed in *E. coli* BL21 (3DE) according to the procedure described by Sambrook *et al*., (1989). The plasmid DNA was isolated and Sequencing was carried out by SeqLab. (Göttingen, Germany). The sequence was available alkane hydroxylase gene present in the EMBL database by using Fasta3 program and analyzed in the GenBank databases with the BLASTN and BLAST programs.

Restriction digestion of pET-15b for ligation: Plasmids were cleaved by restriction enzymes *Nco*I and *Nde*I and dephosphorylated by using Phosphatase alkaline Shrimp according to the method described by Sambrook *et al*., (1989).

Restriction digestion of alcohol dehydrogenase fragment for ligation: The purified *alc*DH fragments from *P. frederiksbergensis*, amplified by PCR were cleaved by restriction enzymes *Nco*I and *Nde*I. DNA fragments were extracted from agarose gel by using NucleoSpin Extract 2 in 1. The purified fragments were kept at –20°C or used directly for DNA ligation.

Ligation of DNA fragment to plasmid arms: The purified *Nco*I/*Nde*I-restricted *alc*DH fragments (*alc*DH//*Nco*I-*Nde*I fragments), were subjected to ligation with purified dephospholyrated *Nco*I/*Nde*I-restricted pET-15b arms (pET-15b//*Nco*I-*Nde*I) by using T4 DNA ligase. Then, the ligation solution was incubated overnight at 4°C.

DNA Sequencing and characterization of the bidirectional gene pair clone: Plasmid DNA was isolated and purified using the method described by Sambrook *et al*., (1989). The gene was sequenced by central Lab. Goettinging, Germany.

Results

Cloning of the complete gene encoding alkane hydroxylase from *P. frederiksbergensis*: The PCR amplification resulted in a 2894 bp fragment from *P. frederiksberghensis* DNA and Southern hybridization was carried out with the PCR derived probe. The expected band size of 2894 bp was obtained (Fig. 1). Nucleotide and amino acid sequences were compared with EMBL, Swiss Port, and GenBank databases using BLAST search which was carried out at the National Center for Biotechnology Information (NCBI). The complete *alk*B gene encoded 491 amino acids with calculated molecular mass of 54.84 kDa. The analysis of the sequenced regions of the *P. frederiksbergensis alk*B revealed the presence of three ORFs which showed similarities to the genes known to play a role in alkane oxidation (Fig. 2).

The alkane hydroxylase gene of *P. frederiksbergensis* has been deposited in the GenBank database under the accession number AY452488.

Phylogenetic analyses of *P. frederiksbergensis* alkane hydroxylase: The Vector NTI Suit v. 6.0 (INFOMAX. INC. 6010 Executive Blvd. No. Bethesda MD20852 USA) program was used to generate a phylogenetic tree of the known alkane hydroxylasegene genes (Fig. 3).

P. frederikesbergensis alkane hydroxylase clustered in a separate group together with the alkane hydroxylase of *R. erythropolis* and formed one phylogenetic branch. Alkane hydroxylase of *P. frederikebergensis* gene has 60% homology to the gene encoding for alkane hydroxylase from *Pseudomonas oleovorans*.

Subcloning and expression of *P. frederiksbergensis* alcohol dehydrogenase gene (*alc*DH): A set of PCR primers was employed to amplify the coding sequence of *P. frederiksbergensis alc*DH. The 5`end of the forward primer was flanked by cleavage site in *Nco*1 and the reverse one flanked by cleavage site in *Nde*1, to allow precise insertion for cloning and expression into vectors pUc19 and pET-15b, respectively. *alc*DH gene of *P. frederiksbergensis* was amplified from pUc19 plasmid containing the 2.9 kb fragment as an insert. Afragment size of 1013 bp was obtained which represented *P. frederiksbergensis alc*DH. Nucleotide and amino acid sequences were compared with EMBL, Swiss Port, and GenBank databases using BLAST search which was carried out at the National Center for Biotechnology Information (NCBI). The complete *alc*DH gene encoded 334 amino acids with molecular mass of 35.79 kDa. The *alc*DH gene sequence of *P. frederiksbergensis* was deposited in the GenBank database (accession number AAR13804).

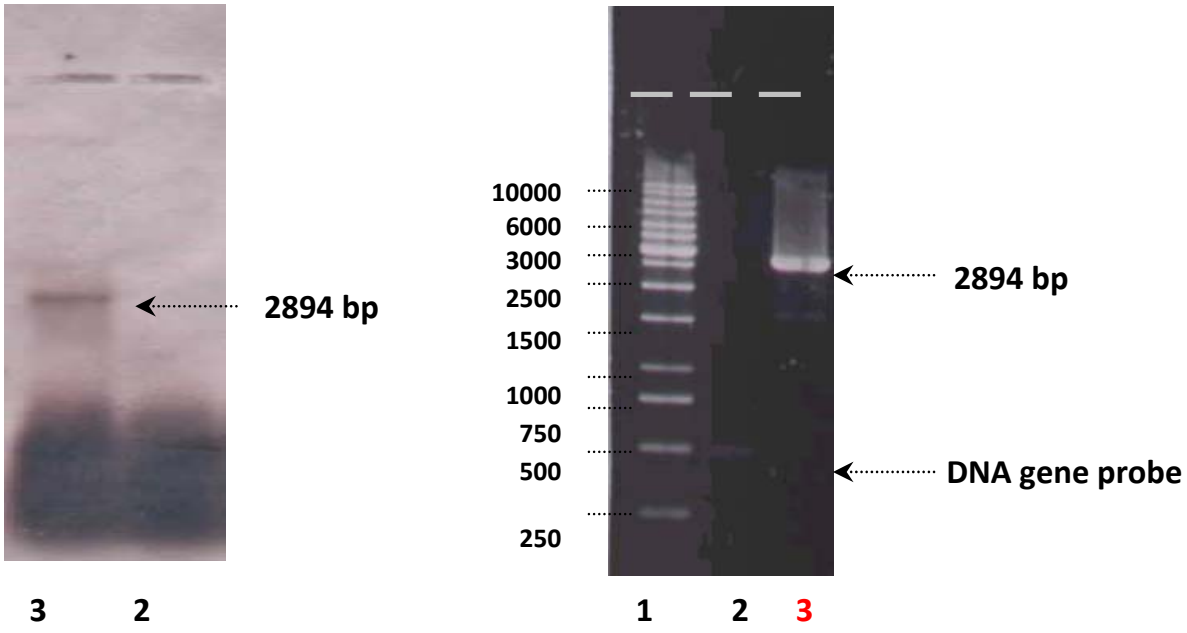

Fig. 1. Southern blotting of *P. frederiksbergensis* alkane hydroxylase gene amplified from PCR
Lane 1. DNA marker (SMO311/2/3)
Lane 2. DNA gene probe (558 bp)
Lane 3. Amplified fragment resulted from (pUc19 and *alk*B primers)

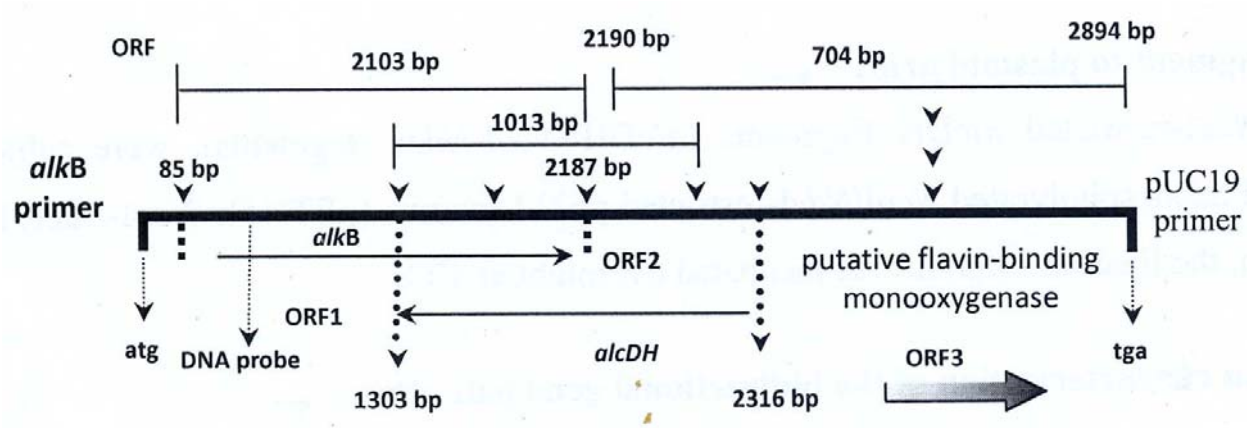

Fig. 2. Genetic organization and analysis of (ORF_S) of *P. frederiksbergensis* allkane hydroxylase. The arrows indicate the direction and translation of the gene.

The translation of this sequence yielded a *alc*DH protein with several motifs. The alignment analysis confirmed that *P. frederiksbergensis alc*DH gene is a member of short chain alcohol dehydrogenase/reductases (SDR). The *P. frederiksbergensis alc*DH had two highly conserved regions near the N-terminal a Thr-GXXXGXG sequence which acts as cofactor binding site and a Tyr-XXX-Lys sequence which acts as active centre.

Phylogenetic analysis of *P. frederiksbergensis* **alcohol dehydrogenase (alcDH):** The Vector NTI Suit v. 6.0 (INFOMAX. INC. 6010 Executive Blvd. No. Bethesda MD20852 USA) program was used to generate a phylogenetic tree of the known alkane hydroxylasegene genes (Fig. 4). The Phylogenetic tree of *P. frederikebergensis* alcohol dehydrogenase showed that this strain is a member of short chain dehydrogenase/reductases family (SDR). The peptide sequence identity between the alcohol dehydrogenases and *P. frederiksbergensis alc*DH ranged from 24 to 45%. The

highest similarity was obtained from *Mycobacterium tuberculosis* alcohol dehydrogenase with 45% identity, followed by *Stryptomyces avemitilis* alcohol dehydrogenase with 44% identity (Fig. 4).

The phylogenetic tree of *P. frederikebergensis* alcohol dehydrogenase showed the that this strain is considered a member of short chain dehydrogenase/reductases family (SDR). The peptide sequence identity between the alcohol dehydrogenases and *P. frederiksbergensis alc*DH ranged from 24 to 45%. The highest similarity was obtained from *Mycobacterium tuberculosis* alcohol dehydrogenase with 45% identity, followed by *Stryptomyces avemitilis* alcohol dehydrogenase with 44% identity.

Discussion

Genetics of alkane degrading enzymes in *P. frederiksbergensis*: Alkane hydroxylase gene of *P. frederiksberghensis* was cloned in order to increase the understanding of the enzyme system involved in aerobic alkane degradation. PCR amplification was based on degenerated primers directed towards the conserved regions of known alkane hydroxylases. The method of amplification was developed by Theo Smits, who was able to show, that many bacteria capable of oxidizing alkanes possess genes related to alkane hydroxylase gene of *P. oleovorans.* All organisms which tested positively by this method belonged to the mesophilic bacteria (Smits *et al*., 1999).

Alignment comparison to the known alkane hydroxylase genes revealed conserved regions corresponding to the eight histidine boxes near the N-terminal, [(RYLWLLGLL); (HELXHK); (EHNRGHH) and (LQRHSDHHA)]. These are highly conserved in all bacterial alkane monooxygenases. The third histdine box is the longest conserved stretch in all alkane hydroxylases, but is not well conserved in other closely related hydrocarbon monooxygenases. An additional well-conserved histidine box (NYLEHYGL), designated the HYG motif is located about 60 amino acids upstream of the third histdine box (Smits *et al*., 1999). This HYG motif is also quite well conserved in related hydrocarbon monooxygenases, such as three xylene monooxygenases (XylM), a nitrotoluene monooxygenase (NtnMa), and two cymene monooxygenases (CymAa) (Smits *et al*., 2002).

Therefore, the third histidine motif and the HYG motif can be used as apparent signature motifs specific for bacterial alkane monooxygenases. Eight histidine act as iron binding ligands (Shanklin *et al.*, 1994). This motif is also conserved among the soluble binuclear iron hydrocarbon oxygenases, such as sMMO and toluene 2-monooxygenase from *Burkhlderia cepacia* G4 (Fox *et al*., 1994). The eight histidine motif is considered a characteristic of nonheme integral membrane desaturases, hydroxylases, oxidases and decarbonylases from prokaryotic and eukaryotic organisms, which are not necessarily related to each other and also occurs in some soluble proteins. The histidyl residues are thought to bind 1 to 3 mol of iron per mol of enzyme as a cofactor and to be part of the active sites of this enzyme (Shanklin *et al*., 1997). The sequence of *alk*B contained conserved stretches of hydrophobic amino acids that span the cytoplasmic membrane many times. In all cases, it was preceded by a stretch of hydrophobic residues which in the *alk*B has been shown to traverse the membrane twice (van Beilen *et al.*, 1992). Another interesting feature of the alignment is the conservation of (HXXXH) sequence motif. This sequence has been shown to bind divalent metals when occuring in an άHelix. This sequence might form a structure similar to the heme tetrapyrole ring and bind iron (van Beilen *et al*., 1994).

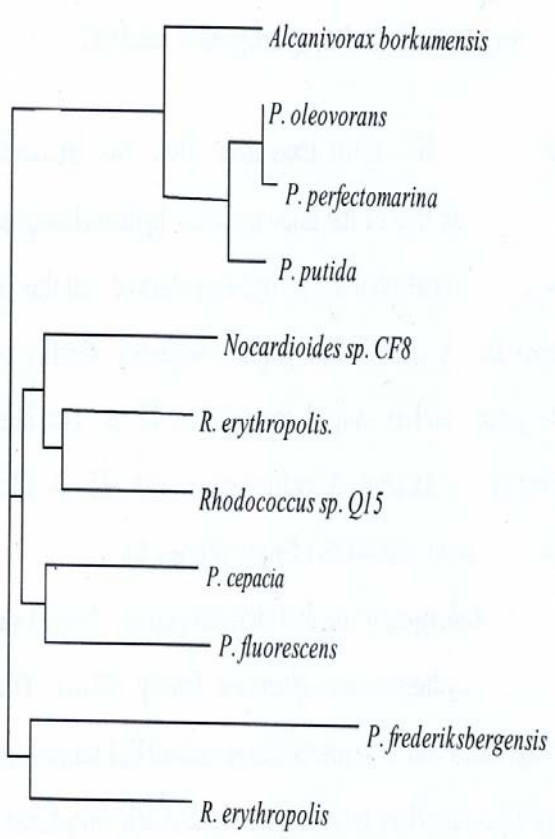

Fig. 3. Phylogenetic tree of *P. frederikebergensis* alkane hydroxylase.

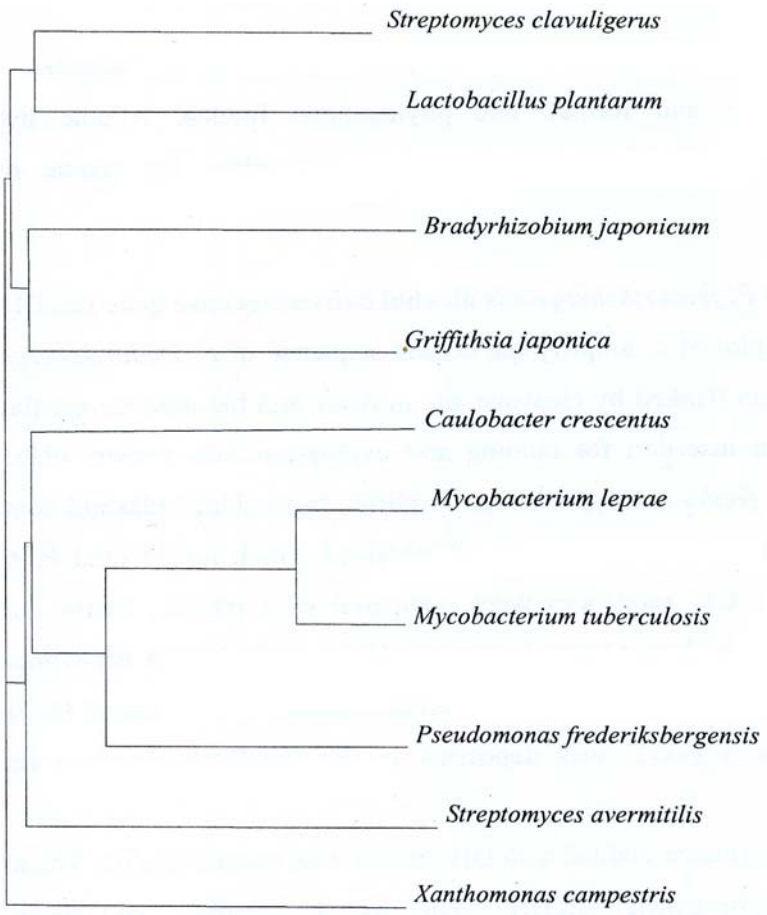

Fig. 4. Phylogenetic tree of *P. frederikebergensis* alcohol dehydrogenase.

The presence of *R. erythropolis* alkane hydroxylase in a separate branch with *R. erythropolis* was surprising, considering that gram positive bacteria are evolutionary very distant from *P. frederiksbergensis*. This similarity between the different alkane hydroxylases was distributed throughout the entire polypeptide, being particularly strong at a series of invariant histidine boxes. These indications suggested that the catabolic gene system described in mesophilic and pyschrotrophs microorganisms like *R. erythropolis* might also occur and function at low temperature in the psychrophilic *P. frederiksbergensis*.

The transfer of biodegradative pathways between mesophiles and psychrorophiles occurred in nature and this hypothesis might be true in case of *P. frederiksbergensis*. Evidence supporting this hypothesis was obtained by demonstrating the successful transfer by conjugation of the TOL plasmid from a mesophilic *P. putida* to a psychrotrophic *P. putida* in which the toluene biodegradative genes were expressed at 0°C. This observation suggests the occurrence of the extensive horizontal gene transfer during evolutionary processes (Meer *et al*., 1992; Zaaijer *et al*., 2007). The alkane hydroxylase found in this psychrophilic organism may have undergone cold adaptation allowing *P. frederiksbergensis* to degrade alkanes at low temperatures more effectively.

The organization of the genes involved in alkane oxidation varies strongly among the different alkane degrading bacteria. The genetic organization of alkane hydroxylase in *P. frederiksbergensis* showed that the genes encoding the enzymes for alkane metabolism were not clustered with *alk*B, *Alk*G and *alk*T which are considered to be the essential components of alkane hydroxylase gene. The alkane hydroxylase system consists of three components: an integral membrane non-heme iron monooxygenase and the two electron transfer components rubredoxin and rubredoxin reductase. Rubredoxin (*alk*G) and Rubredoxin reductase (*alk*T) may be located elsewhere on the *P. frederiksbergensis* chromosome. Previous studies did not prove that *alk*G and *alk*T are involved in alkane degradation in *Acinetobacter* sp., strain ADP1 (Geißdo *et al*., 1995).

In most strains, genes involved in alkane degradation seem to be distributed over the genome. Previous studies showed that none of the rubredoxin reductase genes is located close to an alkane hydroxylase, except the case of *R. erythropolis* (Whyte *et al*., in press), perhaps because they are also involved in other pathways and require a different type of regulation.

Moreover, the alkane hydroxylase gene organization of *P. frederiksbergensis* is different from the genes in *P. oleovrans* alkane hydroxylase. The genes encoding alkane hydroxylase in *P. oleovrans* consists of two rubredoxins, an aldehyde dehydrogenase, an alcohol dehydrogenase, an acyl coenzyme A, synthetase and an outer membrane protein which constitute a single operon (*alk*BFGHJKL) on OCT plasmid (van Beilen *et al*., 1994). In case of *alk* genes in *Acinetobacter* sp., strain ADP 1, the essential genes for alkane degradation are separately located on the chromosome, where *alk*M and *alk*R are located about 369 kb from *alk*T and *alk*G genes encoding rubredoxin and rubredoxin reductase, respectively. Compared to the genes coding for alkane degradation in *P. frederiksbergensis*, the genes are scattered on the chromosome and were not organized in a cluster. The sequence analysis of 2894 bp fragment of the *P. frederiksberghensis* DNA indicated a gene cluster containing alkane monooxygenase (*alk*B) and alcohol dehydrogenase (*alc*DH) genes.

The *P. frederiksberghensis alk*B in the first ORF overlapped partially with the second ORF of alcohol dehydrogenase. This phenomenon is indicative for the translational coupling and is thought to ensure the production of stoichiometric amounts of the involved proteins. Translational coupling has been observed in several rhodococcal operon-like structures from aromatic degradation pathways (Whyte *et al*., 2002).

The gene culster of *P. frederiksbergensis* contained alkane monooxygenase (*alk*B) and putative flavin-binding monooxygenase (hypothetical protein with unknown function) which was oriented in the same direction. Alcohol dehydrogenase (*alc*DH) oriented in the opposite direction. Thus, the organization in *P. frederiksbergensis* may be peculiar. The consequences of such an arrangement for regulation were unclear.

Unfortunately, *P. frederiksbergensis alk*B expression was not successful in *E. coli*. The expression of alkane hydroxylase requires the three componennts *alk*B, *alk*T and *alk*G for the enzyme activity. In case of *Acinetobacter* sp. Strain ADP1, the degradation of alkanes required at least five essential genes (Ratjczak *et al*., 1998). Many possible reasons might be behind the failure to detect the activity of *alk*B.of *P. frederiksbergensis* in *E. coli*. It was possible that the uptake of longer *n*-alkanes requires factors like, porins and alkane-solubilizing compounds that were not produced or expressed in *E. coli*. Furthermore, the unstable nature of hydroxylase component was supposed to be one of the most important reasons for the failure in activity detction. On the other hand, *alk*B may not accept electrons from the rubredoxins reductase in the host strains. In the case of *alk*B from *P. putida* GPo1 the rubredoxin from *E. coli* replaced its *P. putida* GPo1 counterpart (Whyte *et al*., 2002).

Thus, in case of *P. frederiksbergensis alk*B several data were compatible with the idea that the isolated *alk*B was the alkane hydroxylase gene present. First, the PCR amplification strategy to isolate the probe for cloning, was based on degenerated primers directed towards conserved regions of known alkane hydroxylases. Second, the ampified DNA fragment yielded a single hybridization band in southern blots performed with total *P. frederiksbergensis* chromosomal DNA. Thereby, this gene with 60 % homology to *P. oleovorans* was undoubtedly *P. frederiksberghensis* alkane hydroxylase gene.

With respect to *P. frederiksbergensis alc*DH, the theoretical translation of this sequence yielded a protein sequence with several motifs. The alignment comparison confirmed that it was a member of the short chain alcohol dehydrogenase / reductases (SDR). This family of *alc*DH is characterized by N-terminal Thr-GXXXGXG cofactor binding site and Tyr-XXX-Lys active centre motif. The overall similarity among family members is low, usually in the range of 15-30 % identity for nonorthologous proteins. Furthermore, alcohol dehydrogenases catalyze the oxidation of alcohols thereby utilizing NADH as cofactors. In plants, the presumed physiological role for *alc*DH is its reverse reaction, in which *alc*DH reduces acetaldehyde to ethanol, generating NAD^+ from NADH, and thereby allowing glycolysis to maintain sufficient levels of cellular ATP during periods of oxygen deprivation (Gregerson *et al*., 1991). The second role of *alc*DH is to maintain cytoplasmic pH, because ethanolic fermentation, unlike lactic fermentation, does not result in cytoplasmic acidosis.

From phylogenetic analysis point of view, *P. frederiksbergensis* alcohol dehydrogenase had highest similarity to *M. tuberculosis*. This similarity of *M. tuberculosis* alcohol dehydrogenase to *P. frederiksbergensis* was surprising, considering that gram-positive bacteria are evolutionarily very distant from the *P. frederiksbergensis*

group. There were no similarities found towards genes of alkane degrading strains like *Rhodococcus*, *Acinetobacter* and *Pseudomonas.*

In non-viral organisms, the potential advantages of overlapping genes are less clear, although co-regulation may be involved (Istrail *et al*., 2004; Rancure *et al*., 2009). Results of a comparative study of overlapping genes in the genomes of two closely related bacteria revealed that many overlapping genes arise due to incidental elongation of the coding region (Fukuda & Washio, 1999; Kingsford *et al*., 2007). Overlapping genes have generally been thought to be relatively rare in the human genome, but the results of the present study show that they are more abundant than was previously thought. Interestingly, overlapping genes do not appear to be the result of evolutionary pressure to minimize the size of the human genome.

To the best of our knowledge, the phenomenon of overlapping exons is not specific in DNA repair or replication, and further studies are needed to clarify the functional significance of overlapping genes. Clarification of overlapping genes will facilitate the description of roles for each strand of the human genome and will provide insight into the mechanisms of evolution.

It is assumed that new proteins are created by duplication, fusion, or fission of existing coding sequences. Another mechanism of protein birth is provided by overlapping of the genes. These results also suggested that the study such proteins could enhance our knowledge of protein space.

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