JOURNAL OF PURE AND APPLIED MICROBIOLOGY, May 2014. Vol. **8**(Spl. Edn. 1), p. 183-191

Genetics of Alkane Degrading Enzymes by *Pseudomonas frederiksbergensis*

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(Received: 24 January 2014; accepted: 19 March 2014)

P. frederiksbergensis **alkane monooxygenase was cloned in order to increase the understanding of the enzyme system involved in aerobic alkane degradation. The sequence comparisons and ORFS of DNA fragment 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. 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 gene culster of *P. frederiksbergensis* contained alkane monooxygenase and putative flavin-binding monooxygenase (hypothetical protein with unknown function) which was oriented in the same direction. Alcohol dehydrogenase oriented in the opposite direction.**

Key words: Alkane degrading enzymes, *Pseudomonas frederiksbergensis.*

Biotechnological processes for the bioremediation of chemical pollutants offer the possibility of in situ treatments and are mostly based on the natural activities of microorganisms. Biotechnological processes used in the treatment of hazardous wastes offer many advantages over physicochemical processes. When successfully operated, biotechnological processes may achieve complete destruction of organic wastes. However, an important factor limiting the bioremediation of sites contaminated with certain hazardous compounds is the slow rate of degradation (Iwamoto and Nasu, 2001). This slow degradation rate often limits the practicality of using microorganisms in remediating contaminated sites. This is an area where genetic engineering can make a marked improvement. Molecular techniques can be used to increase the level of a particular protein or enzyme or series of enzymes in bacteria with an increase in the reaction rate (Chakrabarty, 1986).

The genetics of aliphatic hydrocarbon degrading microorganisms have been only studied in detail from small number of microorganisms. The best studied system in bacteria is the *OCT* plasmid which codes for a number of proteins involved in growth on (C_6 - C_{10}) *n*-alkanes. Alkane hydroxylase, which is responsible for the biodegradation of aliphatic hydrocarbons is a three component monooxygenase. From biotechnological point of view, alkane hydroxylases are versatile biocatalysts, which carry out a wide range of useful oxidation reactions (Hou *et al*., 1994; Witholt *et al*., 1990). Alkane monooxygenase is comprised of a three polypeptide protein complex that carries

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out alkane degradation. The first gene involved in alkane oxidation is *alk*B, an alkane hydroxylase, which is a cytoplasmic membrane protein. Another gene, *alk*T, a rubredoxin reductase located in the cytoplasm, acts as an electron carrier between NADH and the hydroxylase (Kim *et al*., 2000).

The expression of bacterial catabolic pathways for the assimilation of aliphatic and aromatic hydrocarbons is frequently subject to complex control which links induction of the corresponding genes not only to the presence of the substrates to be degraded but also to the proper physiological status of the cell. This is the case of the alkane degradation pathway encoded by the *OCT* plasmid of *Pseudomonas oleovorans*. The genes of this pathway are grouped into two clusters, *alkBFGHJKL* and *alkST* (Figure 4). The *alkBFGHJKL* operon is transcribed from a promoter, *PalkB*, whose expression requires the transcriptional activator *alk*S and the presence of alkanes. In the absence of alkanes, the *alkST* genes are expressed from promoter *PalkS1*, which is recognized by ó^s-RNA polymerase. Therefore, during exponential growth with a carbon source other than alkanes, the gene for the *alk*S regulator is essentially silent. Upon entry into the stationary phase, when ó^s -RNA polymerase becomes available, transcription of *alkS* from this promoter increases. *alk*S acts as a repressor of *PalkS1*, allowing for low expression of the *alkST* genes. When alkanes are present, the *alk*S protein bound to *PalkS1* activates the expression of promoter *PalkS2*, located 38 nucleotides downstream from *PalkS1*, and provides for high expression of the alkST genes (van Beilen *et al*., 2002).

Therefore, present work was to understand the Genetics of alkane degrading enzymes by *Pseudomonas frederiksbergensis* in order to increase the understanding of the enzyme system involved in aerobic alkane degradation.

MATERIALS AND METHODS

Growth of the organisms

P. frederiksbergensis was isolated from in earlier work of Abdel Megeed (2014). Isolated DNA from *P. frederiksbergensis* was dissolved in TE buffer and stored at –20°C for further experiments according to Pither *et al*., (1989).

Detection and amplification of alkane hydroxylase of *P. frederiksbergensis*

P. frederiksbergensis was screened for the presence of the gene encoding alkane hydroxylase (*alk*B). The amplification strategy of the gene was based on degenerated specific primers as described by Innis *et al*., (1990). The used oligonucleotides primers which were derived from *P. oleovorans* ATCC 29347. These oligonucleotides had the following sequence: Forward : 5'-TGGCCGGCTAC TCCGATGATCGGAATCTGG-3' and Reverse: 5'-CGCGTGGTGATCCGAGTGCCGCTGAAGGTG-3'. The amplification was used for isolation of gene probe for (*alk*B) cloning. This amplification with *P. frederiksbergensis* DNA was carried out according to the standard procedures using a thermal cycles (Abdel-Megeed, 2014). PCR fragments were analyzed and visualized by gel electrophoresis (0.8 %) using Sybr^{â} Green (Biozyme, Germany). The fragments were purified from the gel and used again as DNA template by PCR and sent to be sequenced after purification from the gel. Sequencing was carried out by SeqLab (Göttingen, Germany). 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.

Southern blotting for hybridization and alkane hydroxylase detection and localization

To check the amplification of the correct PCR fragments, Southern hybridization was performed for the detection of the gene encoding alkane hydroxylase (*alk*B). Southern blot for hybridization was carried out using Hybrid^{TM}N^{+} (membrane optimised for nucleic acid transfer). Restriction of genomic DNA was performed with the following endonucleases: *Eco*R1, *Nde*1, *Nco*1, *Bam*1 and *Eco*881 according to the instructions described by Sambrook *et al*., (1989). All buffers and enzymes were from Fermentas, Roche, NEB and Pomega. DNA gel electrophoresis was performed according to standard procedures (Sambrook *et al*., 1989) and the gel was prepared with 1 % (w/v) agarose dissolved in TBA. The run was at 70 volt. The gel was stained with Sybr^{â} Green (Biozyme, Germany).

Digoxigenein labeled alkane hydroxylase gene probe

Digoxigenein (DIG) is a steroid haptane derived from the plant *Dgitalis purpure*. The digoxigenin is linked via a spacer arm to the nucleotide deoxyuridine triphosphate (dUTP) to give DIG-dUTP. Random hexanucleotides serve as primer for Klenow DNA polymerase. Digoxigenin molecules are incorporated approximately every 20 bases. Target DNA was made available for hybridization by being immobilized onto a nylon membrane. Hybrid was detected using an anti digoxigenin antibody conjugated to the enzyme alkaline phosphate. The *alk*B gene probe was prepared by using the PCR-DIG-Labeling Kit of Roche.

Transfer of DNA to membrane

The digested DNA of *P. frederiksbergensis* was blotted upon the positively charged nylon membrane and fixed by UV radiation for 5 min. Hybridization with gene probe was performed over night at 65°C. Stringency washing was carried out at the same hybridization temperature. The detection of hybridized membrane was done according to the procedure described by Sambrook *et al*., (1989). After detection and development, the membrane was incubated in plastic bag containing staining solution in dark for at least 4 hours. After the development, the reaction was stopped by washing the membrane in distilled water to avoid the background noise.

Cloning and sequencing of *P. frederiksbergensis* alkane hydroxylase gene

The genomic DNA of *P. frederiksbergensis* was digested with the appropriate restriction enzyme to generate compatible ends (*Eco*R I) according to the standard method described by Sambrook *et al*., (1989). The gel was prepared with 0.8 % (w/v) agrose dissolved in TBA. The run was carried out at 70 volt. The gels were stained with Sybrâ Green (Biozyme, Germany). The correct restriction fragments "with 3` and 5` overhangs or sticky ends" were cut, purified from agarose gel and stored at –20°C for further experiments.

Preparation of the pUc19 vector

The vector was digested with the same restriction enzymes mentioned in 2.5.4.1 and incubated over night. The gel was prepared as previously mentioned. The digested plasmid was linearized and dephosphorylated with CIP (New England Biolabs) according to the method described by Sambrook *et al*., (1989). Then, if not sated otherwise, the digested vector was stored at –20°C till used. The DNA fragments and the vectors were ligated with the presence of T4 DNA ligase (Invitrogene) following the instructions of the manufacturer and the standard procedure of Sambrook *et al*., (1989). The ligation solution was incubated over night at 4°C. For precipitation of plasmid DNA in order to use it as a DNA template in PCR, 6 volume of isopropanol were added and the sample was incubated at RT for 10 min, before it was centrifuged at max speed for 10 min at RT. The supernatant was discarded and 100 µl of 2 *M* ammonium acetate (pH = 7.4) were added to the pellet, vortexed and incubated on ice for 5 min. Subsequently the sample was centrifuged again for 5 min at RT. The supernatant was transferred to a fresh tube and mixed with 100 µl of isopropanol before it was incubated again on ice for 10 min. The sample was centrifuged at maximum speed for 10 min at RT and the resulting pellet was washed with ice cold ethanol (70 %), centrifuged and vaccuum dried. The plasmid pellet was dissolved in 50 µl of TE buffer and stored at –20°C. Alkane hydroxylase amplification and Southern blotting was carried out as described by Sambrook *et al*., (1989).

Sequencing and analysis method

Sequencing was carried out by SeqLab. (Göttingen, Germany). Sequencing results were compared to the available alkane hydroxylase genes present in the EMBL database by using Fasta3 program and analyzed also in the GenBank databases with the BLASTN and BLAST programs.

Subcloning and expression of alcohol dehydrogenase gene

The pET-system is considered one of the powerful systems developed for the cloning and expression recombinant proteins *E.coli*. The pET-15b vector carries an N-terminal His.Tag® sequence followed by a thrombin site and three cloning sites. The cloning and expression regions of the coding strand transcribed by T7 RNA polymerase. The plasmid carries an ampicillin resistance for selection and a part of ß-galactosidase gene for blue white screening.

Alcohol dehydrogenase gene (alcDH) amplification by PCR

Amplification of *alc*DH from *P. frederiksbergensis* by PCR was carried out in reaction mixtures consisting of 75 pmol of the following primers: Forward: 5'-TGTATACCATGG AAGCAACAGGGCTACTAGCGTACTGC-**3** and Reverse: 5'-GCGGCCATATGCTATTATTG AGAACTATTCAACGGCCG-**3'**. 200 nmol of deoxynucleoside triphosphates, 0.2 U of DNA polymerase and 1 to 100 ng of DNA template (cDNA from *P. frederiksbergensis*) in a final volume of 50 µl were mixed well. The amplification was run according to the following program:

Restriction digestion of pET-15b for ligation

Plasmids were cleaved by restriction enzymes NcoI and NdeI 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. Preparation of competent Cells was carried out according to the method described by Abdel-Megeed, (2004). After overnight incubation period, colonies selected by antibiotic resistance were picked from agar plates with sterile toothpick sticks. The colonies were transferred and cultivated in LB medium containing ampicillin (50 mg/ml) and IPTG (100 µl/ml). The vials were incubated over night at 37°C and 250 rpm. Plasmids were isolated from each positive colony that hybridized with the DNA probe according to the standard method described by Sambrook *et al*., (1989). They were digested with *Nde*I and *Nco*I and DNA fragment and separated using gels electrophoresis. The run was performed at 77 volt. The gels were stained with Sybr[a] Green. Plasmids sample containing alcDH fragment was selected to transform into competent host cells for protein expression. The recombinant plasmids carrying alcDH fragment were transformed into E. coli BL21 (3DE) competent cells. As a negative control, transformation of plasmids pET-15b without insert into the host cells were also performed. The grown colonies on LB-amp. plates were used for crude extract preparation and alcDH activity assay. For expression analysis, 200 ml of LB medium containing 100 µg/ml ampicillin were inoculated with 1 ml of pro-culture of *E. coli* BL21 (3DE) cells harboring the respective gene. The cell mixtures were incubated at 37°C and 250 rpm till an OD of 0.6 was reached. Expression was initiated by the addition of IPTG after 3 hours of cultivation. Recombinant cells were grown in 1 L-Erlenmayer flask filled with 300 ml LB medium containing 100 µg/ml ampicillin in the absence of IPTG. The medium in every flask was inoculated with 20 ml of preculture with OD_{600} of 0.4. Then they were incubated over night at 37°C and 250 rpm. The cultivated cell culture was centrifuged at 4°C with 15,000 rpm for 20 min. The resulting cells pellet were washed with 50 m*M* sodium phosphate buffer and stored at –20°C.

RESULTS AND DISCUSSION

Detection of the gene encoding alkane hydroxylase in *P. frederiksbergensis*

Search for sequences homologous to other alkane hydroxylases in the genomic DNA of *P. frederiksbergensis* was carried out by PCR using highly degenerated oligonucleotides based on the highly conserved sequence motifs from *P. oleovorans* (Smits *et al*., 2002). Three DNA fragments of 230, 558, and 2894 bp were amplified from *P. frederiksbergensis* genomic DNA (Figure 30). The sequence of 558 bp fragment showed high similarities to known alkane hydroxylases. The highest score was obtained for the alkane hydroxylase from *P. oleovorans* ATCC 29347 with 60 % identical amino acids. The amplified DNA fragment of 558 bp was used for cloning the alkane hydroxylase gene from *P. frederiksbergensis* genomic DNA.

Hybridizing of *P. frederiksbergensis* digested DNA with the PCR derived probe

Genomic DNA *P. frederiksbergensis* was digested with the following endonucleases: EcoR1, Nde1, Nco1, Bam1 and Eco881. The digested DNA

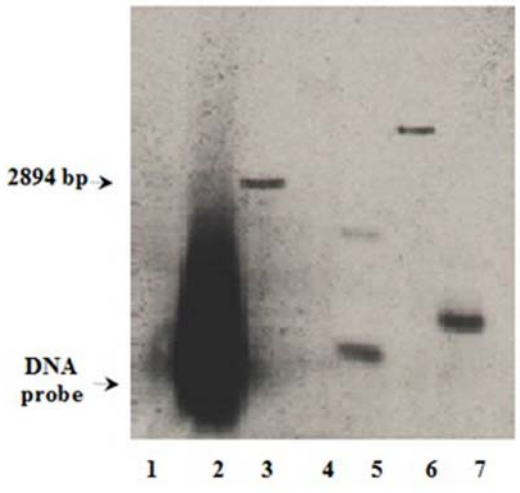
was blotted upon the positively charged nylon membrane and fixed by UV. The membrane was hybridized with the PCR derived probe. Hybridization resulted in different bands for the different digests (Fig. 1).

Cloning of the complete alkane hydroxylase gene from *P. frederiksbergensis*

For the amplification of whole alkane hydroxylase gene of *P. frederiksberghensis*, the combination between specific *alk*B primers and pUc19 primer were used. The PCR amplification resulted in a 2894 bp fragment. To check the fragment obtained from *P. frederiksberghensis* DNA amplification, Southern hybridization was carried out with the PCR derived probe. The expected band of 2894 bp was obtained (Fig. 2).

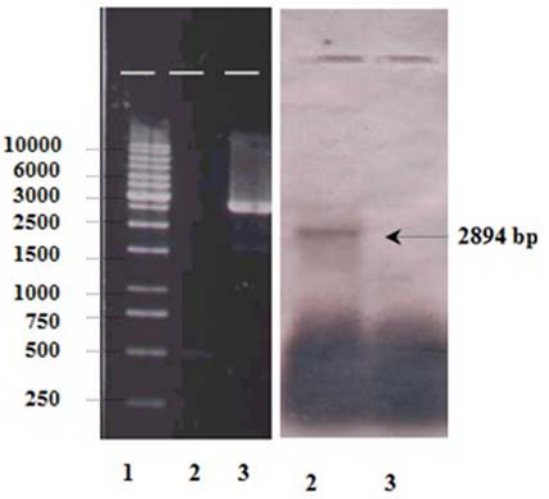
Nucleotides sequence analysis and accession number of the alkane hydroxylase gene

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



Lane 1. 1kb DNA ladder (SMO311/2/3); Lane 2. DNA gene probe ; Lane 3. *Eco*R I; Lane 4. *Nde*I; Lane 5. *Nco*I; Lane 6. *Ava*I; Lane 7. *Bam* I

Fig. 1. Detection and localization of alkane hydroxylase by southern hybridization



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. Southern blotting of *P. frederiksbergensis* alkane hydroxylase gene amplified from PCR

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 showing similarities to genes known to play a role in alkane oxidation (Abdel-Megeed, 2014). The alkane hydroxylase gene of *P. frederiksbergensis* has been deposited in the GenBank database under the accession number AY452488. The first big ORF encoding *alk*B contained several conserved regions corresponding to the eight histidine boxes near N-terminal, [(RYLWLLGLL); (HELXHK); (EHNRGHH) and (LQRHSDHHA)], and an additional well-conserved histidine box (NYLEHYGL). (Figure 34). The sequence of *alk*B contained conserved stretches of hydrophobic amino acids. Another interesting feature of the alignment is the conservation of (HXXXH) sequence motif. The second ORF encoded alcohol dehydrogenase (*alc*DH). The third ORF contained a protein with unknown function. The sequence obtained was aligned with other known hydroxylase genes (Figure 34). 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 strategy to isolate gene probe for cloning 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*., 200). 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 ÜHelix. This sequence might form a structure similar to the heme tetrapyrole ring and bind iron (van Beilen *et al*., 2003). 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). 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 (Fig. 3) (van Beilen, 2003).

Based on the sequence comparisons and ORFS results, the regulation and the organization of genes involved in alkane oxidation in *P. frederiksberghensis* varied also from 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

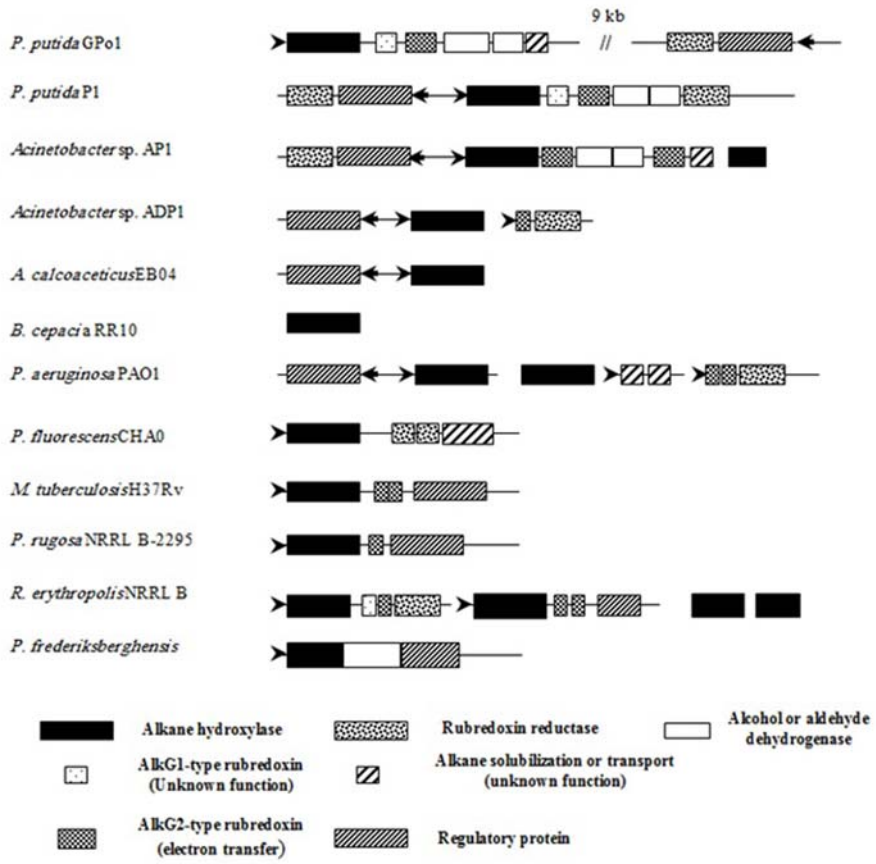


Fig. 3. Organization of *alk* genes in different microorganisms. The arrows indicate the direction of transcription van Beilen, (2003)

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* perhaps because they are also involved in other pathways and require a different type of regulation. 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*.

CONCLUSION

To gain a clear understanding for the alkane monooxygenase genetic organization of *P. frederiksbergensis*, the gene was cloned. The sequence comparisons and ORFS of DNA fragment 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.

ACKNOWLEDGEMENTS

The authors extend their appreciation to the Deanship of Scientific Research at King Saud University for funding this work through research group no RGP-010.

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