ABSTRACT

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Hydrocarbon degrading bacteria and fungi are widely distributed in nature and they are capable of assimilating hydrocarbons of almost all kinds, starting from short chain alkanes to higher aliphatic and aromatic compounds. Studies on alkane degradation have shown that microbial degradation of n-alkanes normally proceeds by a monoterminal attack by monooxygenase, usually a primary alcohol is formed followed by an aldehyde and then a monocarboxylic acid, which is further degraded by β-oxidation. Among the gaseous aliphatic hydrocarbon utilizers, methanotrophs have been studied extensively. *Pseudomonas oleovorans*, utilizing a medium chain length aliphatic hydrocarbon octane has also been studied in great detail and different components involved in octane metabolism and its regulation have been characterized. In contrast, studies on medium chain gaseous hydrocarbon (ethane, propane and butane) utilizers have been very few and as a result, information regarding the mechanism of utilization of these compounds at physiological or genetic level is limited and fragmentary.

Microbial degradation of aromatic compounds may involve mono and dioxygenases. Action of a dioxygenase results in the formation of a diol, which is followed by an oxidative cleavage of the benzene ring and formation of a diacid. This diacid is finally converted by successive enzymatic steps into precursors of β-oxidation pathway. Formation of para-hydroxybenzoate (*p*-OHB) and 3,4 protocatechuate as intermediates is a very common event in aromatic hydrocarbons utilization pathways. The enzyme responsible for hydroxylation of *p*-OHB has been studied extensively and acts as a prototype for studying various flavooxygenases.

In order to investigate the mechanism of gaseous alkane utilization, a few bacteria were isolated from Gujarat oil fields, which were able to grow on propane or butane as sole source of carbon and energy. Two of these bacteria were tentatively identified on the basis of biochemical and physiological characteristics as *Pseudomonas* sp., designated as IMT37 and IMT40 and another microorganism was identified as *Rhodococcus* sp. IMT35. However, the identification of these microorganisms to the species level was not possible by classical approaches. Initial studies on these microbes showed that at least four polypeptides (58, 42, 32, 27 kDa) were induced specifically when these cultures were grown on gaseous hydrocarbons. Antibody against the 58 kDa polypeptide was used to isolate a 4.9 kb DNA fragment having an ORF for a 52.4 kDa protein. This ORF has been

shown to be involved in the first step of alkane utilization by insertional inactivation studies. Antibody against another protein of 32 kDa was also used to identify a clone having a 4.1 kb insert. Detailed characterization of the ORF and the 4.1 kb DNA fragment and their roles in hydrocarbon utilization pathway was lacking.

In order to fill the gap in our understanding about the organism and mechanism of hydrocarbon utilization, this study was planned with the following objectives:

(a) Molecular characterization of hydrocarbon utilizing *Pseudomonas* strains,

(b) Characterization of the clone identified with the anti-32 kDa antibody and

(c) Detailed characterization of the ORF coding for the 54 kDa polypeptide involved in butane utilization.

This thesis begins with an introductory chapter including a review of literature in related fields. Chapter 2 describes the materials used and methods employed in this investigation.

In Chapter-3, it has been shown that a polyphasic approach is very useful in determining true identity of the bacteria used in this study. It presents the detailed characterization of two gaseous alkane-utilizing bacteria using biochemical and physiological tests, fatty acid profile as well as modern genotypic methods. Results from biochemical and physiological tests showed that the characters of these two strains match with the members of the genus *Pseudomonas* but placing them in any species was not possible. Among various species of *Pseudomonas*, these two bacteria were found to be closer to *Pseudomonas mendocina* than any other species. Fatty acid profile of these two strains also suggested similarity to *Pseudomonas mendocina* followed by *Pseudomonas stutzeri*. DNA-DNA hybridization studies with genomic DNAs of related type species of the genus *Pseudomonas* including *Pseudomonas mendocina* did not show enough relatedness to warrant their inclusion in any existing species. Finally, 16S rRNA gene sequencing followed by a phylogenetic tree construction showed that these two strains are closely related to each other but clustered separately from all existing species of this genus. Hence, a new species name *Pseudomonas indica* was proposed for these two bacteria. IMT40 was designated as a strain of above species as it differed from IMT37 in a few physiological and biochemical tests.

Chapter-4 describes the detailed characterization of a clone identified by immuno-screening with anti-32 kDa antibody. This clone (pTA1) contains a DNA fragment of 4.1 kb from *Pseudomonas* sp IMT40. Role of this DNA fragment and of adjoining region has been investigated in this chapter. Initial partial DNA sequencing of this insert showed

homology with *p*-hydroxybenzoate hydroxylase of few microorganisms. *Pseudomonas* sp. IMT40 could grow on para-hydroxybenzoate (*p*-OHB) as a sole source of carbon and energy with a generation time of 150 minutes at 37 °C compared to generation doubling time of 44 minutes when grown on LB.

A detailed restriction map of pTA1 was constructed. A number of sub clones were constructed from pTA1 for complete sequencing of the insert region and adjoining region. Total length of insert in pTA1 was determined to be 4307 bp containing four complete genes *pob*R, a regulator for *p*-hydroxybenzoate hydroxylase; *pcaQ*, a regulator for protocatechuate 3,4 dioxygenase; *pcaHG*, subunits of protocatechuate di-oxygenase and a partial ORF coding for N-terminal of *p*-hydroxybenzoate hydroxylase (*pobA*).

A kanamycin gene cassette was used to inactivate the genes present in this DNA fragment at four different places. These disruption clones were linearized by restriction digestion and were used to transform the wild-type strain of *Pseudomonas* sp. IMT40 by electroporation. In three cases, none of the kanamycin resistant transformants could utilize *p*-OHB or 3,4 protocatechuic acid (next intermediate in *p*-OHB utilization pathway) indicating their involvement in *p*-OHB utilization. In the fourth case, all the kanamycin resistant transformants could grow on *p*-OHB as well as protocatechuic acid. Among all the transformants obtained from four different types of disruption clones, 25 % of the transformants lost their ability to grow on butane as well. It indicated involvement of some common component(s) in butane utilization pathway and *p*-OHB utilization. To confirm it, butane monooxygenase (BMO) activity in *p*-OHB grown cells and para-hydroxybenzoate hydroxylase (PHBH) activity in butane exposed cells was determined. Results from this experiment indicated cross-induction of above two enzymes. So far, there have been no reports on an organism capable of utilizing both gaseous alkanes and *p*-OHB.

In order to get the complete *pobA* gene, flanking regions around this 4307 bp insert was cloned from IMT40 and sequenced. Complete DNA sequence of 7171 bases (GenBank accession no. AF302797) including 4307 nucleotides from pTA1 and flanking region had five ORFs whose functions could be assigned. In addition, there was another partial ORF, which could code for a 394 amino acids (42.8 kDa) long polypeptide showing low level of homology with a malonate transporter protein (MdcF) and a few hypothetical proteins in the database. Besides this, upstream to stop codon of PHBH, there was another partial ORF, which showed homology with ferulyl-CoA synthetase sequences. Although the genes involved in *p*-OHB utilization in IMT40 had homology with corresponding