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A novel gene encoding a 54 kDa polypeptide is essential for butane utilization by *Pseudomonas* sp. IMT37

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Twenty-three propane- and butane-utilizing bacteria were isolated from soil samples collected from oilfields. Three of them have been identified as *Rhodococcus* sp. IMT35, *Pseudomonas* sp. IMT37 and *Pseudomonas* sp. IMT40. SDS-PAGE analysis of the membrane of *Rhodococcus* sp. IMT35 revealed the presence of at least four polypeptides induced by propane. Polyclonal antibody raised against a 58 kDa polypeptide from *Rhodococcus* sp. IMT35 specifically detected bacteria which were actively utilizing propane or butane. Immunoscreening of a genomic library in λgt11 with this antibody resulted in isolation of a clone containing a 4·9 kb *Eco*RI genomic DNA fragment. This 4·9 kb DNA fragment was found to hybridize specifically with organisms which could grow on propane or butane. This fragment could therefore be used as a probe for detection of such bacteria. A 2·3 kb fragment having an ORF encoding a polypeptide of 54 kDa was identified by screening a genomic library of *Pseudomonas* sp. IMT37 with this 4·9 kb *Eco*RI fragment. The sequence of the ORF (designated *orf54*) was found to be novel. Primer extension and S1 nuclease mapping showed that transcription of the ORF starts at base 283 and it had sequences upstream similar to that of a *Pseudomonas* promoter (−12, −24 type). Disruption of the ORF by a kanamycin ('kan') cassette prevented the organism from growing on any alkane but did not affect its ability to utilize the respective alkanols and acids, indicating that alcohol dehydrogenase and subsequent steps in the pathway remained unaltered. The mutants had no detectable level of butane monooxygenase activity. Therefore, the product of this gene plays a crucial role in the first step of the pathway and is an essential component of monooxygenase. The findings imply that this bacterium either employs a common genetic and metabolic route or at least shares the product of this gene for utilization of many alkanes.

Keywords: alkane utilization, butane monooxygenase, primer extension, S1 nuclease mapping, insertional inactivation

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Abbreviations: BMO, butane monooxygenase; LPG, liquefied petroleum gas; MMO, methane monooxygenase; pMMO, particulate MMO; PMO, propane monooxygenase; sMMO, soluble MMO.

The GenBank accession number for the *orf54* sequence is L81125.

INTRODUCTION

Gaseous hydrocarbons have long been known to act as sole source of carbon and energy for many bacteria and for a few yeasts and fungi (Miyoshi, 1895; Söhngen, 1906; Lukins & Foster, 1963; Coleman & Perry, 1985; Woods & Murrell, 1989; Saeki & Furuhashi, 1994). Among the gaseous alkanes, methane, propane and butane are the primary substrates which are metabolized by these micro-organisms. A number of review articles on the ecology, physiology and genetics of methanotrophs have appeared in the literature (Quayle & Ferenci, 1978; Colby *et al*., 1979; Dalton, 1980; Hanson, 1980; Quayle, 1980; Higgins, 1980; Dalton *et al*., 1984; Lidstrom & Stirling, 1990). Usually the presence and relative abundance of methane, propane and butane in subsoil are indicative of petroliferous regions. It is, therefore, reasonable to assume that micro-organisms capable of utilizing gaseous hydrocarbons will be present in relative abundance in petroliferous regions compared to non-petroliferous regions and such correlations using methane utilizers were attempted previously (Taggart, 1967; Sealy, 1974; Lonsane *et al*., 1977). Methods proposed earlier were time-consuming and may not be easy to perform in field conditions. One objective of our study was to find out if the presence of propane- or butane-utilizing bacteria could be detected rapidly and unambiguously from environmental samples. Since methane could also be of recent geological origin, methane-utilizing bacteria were not considered in our investigation.

The conventional method for detection of specific micro-organisms is selective plating. Although easy to use, this method takes time and can cover only limited types of bacteria, and selection, being a growth-dependent process, may miss out organisms which require different media or temperatures. Polyclonal and monoclonal antibodies have proved to be more reliable and easy to use for detection of target organisms. However, this method of detection depends on the product(s) of a gene(s) which may or may not be expressed depending on the environmental conditions the microbes encounter. Another approach is the use of nucleic acid probes because they can recognize target sequences at any stage of growth, even if specific micro-organisms are in low abundance, and unlike immunological probes do not require expression of a specific gene(s). Function-specific DNA probes whether based on DNA hybridization or PCR amplification can detect a range of microbes irrespective of their taxonomic affiliation (Grunstein & Hogness, 1975; Torsvik, 1980; Sayler *et al*., 1985; Ogram *et al*., 1987; Holben *et al*., 1988).

In order to develop a function-specific probe, it is necessary to identify one or more novel properties shared by the target micro-organisms. The first crucial step in the oxidation of alkanes is catalysed by monooxygenases. Among the various alkane monooxygenases known, methane monooxygenase (MMO) is the best studied to date. The soluble (sMMO) and particulate (pMMO) MMOs have been purified and characterized (Colby *et al*., 1977; Fox & Lipscomb, 1988; Fox *et al*., 1988, 1989; Green & Dalton, 1989; Woodland & Dalton, 1984; Stainthorpe *et al*., 1990; Semrau *et al*., 1995). MMO is a multicomponent enzyme consisting of a hydroxylase, a coupling protein and a reductase. A crystal structure of the hydroxylase component of MMO has been elucidated (Rosenzweig *et al*., 1993). The alkane monooxygenase from *Pseudomonas oleovorans*, like MMO, is also a multicomponent enzyme and consists of *alkB*, *alkG* and *alkT* gene products – alkane hydroxylase, rubredoxin and rubredoxin reductase, respectively (Kok *et al*., 1989a, b; Eggink *et al*., 1987a, b, 1988, 1990).

In propane and butane metabolism, the first and the key step is presumably catalysed by propane and butane monooxygenase (PMO and BMO), respectively. The presence of PMO has been shown in *Rhodococcus rhodochrous* PNKb1 but the enzyme has eluded purification because of its unstable nature (Woods & Murrell, 1989) and characterization has not been possible. PMO and BMO may also be multicomponent enzymes. Based on biochemical evidence and product accumulation, a pathway for butane metabolism in *Nocardia* TB1 (Van Ginkel *et al*., 1987) and '*Pseudomonas butanovora*' (Arp, 1999) has been proposed which in both organisms appears to be very similar. Comparative physiological studies using three butane-grown bacteria, '*P. butanovora*', *Mycobacterium vaccae* JOB5 and an environment isolate CF8, led to the conclusion that there is diversity in BMOs (Hamamura *et al*., 1999). The genetic organization of pMMO (Semrau *et al*., 1995) and sMMO (Stainthorpe *et al*., 1990) is now known. However, virtually no information is available about the biochemical, genetic and molecular basis of C_2–C_5 alkane metabolism.

In this paper, we report the isolation of three gaseous-alkane-utilizing bacteria and describe the identification of a 58 kDa polypeptide induced by butane. Polyclonal antibody raised against this polypeptide was used to detect butane-utilizing bacteria and for identification of a 4·9 kb DNA fragment containing the gene encoding this protein. This DNA fragment could also be used as a probe for specific detection of propane- and butane-utilizing bacteria. The gene encoding this 58 kDa protein has been characterized and its role in butane and higher alkane utilization has been established in a facultative butanotroph, *Pseudomonas* sp. IMT37.

METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids are listed in Table 1 and Table 2, respectively.

Materials. Butane, propane, hexane, octane, nonane and decane were purchased from Aldrich and Matheson Gases. Liquefied petroleum gas (LPG) was obtained from the Oil and Natural Gas Commission (ONGC), India. Zero air was purchased from Indian Oxygen. Freund's complete and incomplete adjuvants were purchased from Difco Laboratories. Acrylamide, agarose, ethidium bromide, DTT,

Table 1. Bacterial strains used in this study

MTCC, Microbial Type Culture Collection & Gene Bank, Chandigarh (India); NCIB, National Collection of Industrial Bacteria, UK.

Micro-organism	Propane/butane utilization
Arthrobacter viscosus sp. (MTCC 22)	−
*Bacillus subtilis** (MTCC 121)	−
Corynebacterium liquefaciens (MTCC 25)	−
Escherichia coli JM109	−
Escherichia coli MC1061	−
Escherichia coli (MTCC 131)	−
*Escherichia coli** (MTCC 118)	−
Flavobacterium antarcticus (MTCC 675)	−
Gluconobacter oxydans (MTCC 904)	−
Lactobacillus fermentum (MTCC 903)	−
Micrococcus roseus (MTCC 678)	−
Mycobacterium sp. (MTCC 290)	−
Nocardia petroleophila (MTCC 273)	−
Pseudomonas cepacia (MTCC 438)	−
*Pseudomonas putida** (MTCC 102)	−
Pseudomonas sp. (MTCC 129/NCIB 11309)	G†
Pseudomonas sp. IMT37	+
Pseudomonas sp. IMT40*	+
Rhodococcus rhodochrous (MTCC 289) (J. J. Perry, USA)	H†
Rhodococcus sp. IMT35*	+
Serratia marcescens (MTCC 97)	−
Vibrio sp. (MTCC 866)	−
*Xanthobacter autotrophicus** (MTCC 133)	−
*Zymomonas mobilis** (MTCC 88)	−
Cultures yet to be identified: IMT14, IMT21, IMT23, IMT24, IMT32a and b, IMT33, IMT34, IMT39, IMT41	+

* These strains were used for dot-ELISA.

† These strains are reported as natural gas (G) and hydrocarbon (H) utilizers, respectively.

Table 2. Plasmids used/constructed during this study

Plasmid	Properties	Reference
pUC19	Amp^r, ColE1 replicon, plasmid of 2·69 kb	Yanisch-Perron *et al.* (1985)
pHC79	Amp^r Tet^r, pMB1 replicon, cosmid of 6·4 kb	Hohn & Collins (1980)
pRT1–pRT7	pHC79-based plasmids carrying inserts of 6–26 kb from *Pseudomonas* sp. IMT37 which shows positive signal with 4·9 kb fragment	This study
pRT3A	Subclone of pRT3 in pUC19 carrying 2·3 kb *Kpn*I–*Hin*dIII fragment	This study
pRT3B	Subclone of pRT3 in pUC19 carrying 3·7 kb *Kpn*I–*Hin*dIII fragment	This study
pRT3A.1–pRT3A.10	Subclones of pRT3A in pUC19 constructed for sequencing	This study
pRT3B.1	Subclone of pRT3B carrying 0·3 kb *Kpn*I–*Pst*I fragment	This study
pRT3AΔA–pRT3AΔJ	Deletion subclones of pRT3A in pUC19 constructed for sequencing	This study
pTC4	pUC18 carrying the 4·9 kb fragment as insert	This study
PGEMPstRT3	pGEM5Z carrying the 1·4 kb *Pst*I fragment of pRT3	This study

EDTA, formamide, lysozyme, proteinase, RNase, TEMED, SDS, ampicillin, tetracycline, kanamycin and streptomycin were purchased from Sigma. Nylon membranes were purchased from Amersham; IPTG, restriction endonucleases and other DNA-modifying enzymes from Promega, Boehringer Mannheim, New England Biolabs, Stratagene; sequencing kits (Sequenase version 2.0) from United States Biochemicals and X-ray films from Hindustan Photo Films. Unless specified otherwise, analytical grade chemicals from commercial sources were used.

Isolation of propane- and butane-utilizing bacteria. Soil samples were collected from known oilfields of Gujarat, India. One gram of soil was suspended in 10 ml mineral salt medium (Whittenbury *et al*., 1970) in 50 ml vials fitted with gas-tight closures and then crimped. The vials were filled with LPG (55%, v/v, propane; 45%, v/v, butane; minor amounts of butene, propene and mercapton) and then incubated at 30 °C for 3–4 d on an orbital shaker at 200 r.p.m. Subculturing was carried out for five cycles in mineral salt medium with LPG as sole source of carbon. Hydrocarbon gases (LPG, butane, propane, ethane and methane) were always used as a mixture with air in a ratio of 40:60. Serial dilutions of the final cultures were then spread on mineral medium agarose (MMA; 2%, w/v) plates. The plates were placed in a desiccator, which was then filled with LPG and incubated at 30 °C. After 7 d incubation, colonies from these plates were picked up at random and streaked on MMA plates for isolation of single colonies. The purified single colonies were replica-plated onto MMA and their growth was checked on propane, butane and air in the presence and absence of KOH. This combination of growth conditions was used to avoid the selection of CO_2 fixers.

Growth studies. For growth on propane and butane, cells were streaked on MMA plates and were placed in a desiccator. The desiccator was evacuated and then filled with a propane or butane and air mixture (40:60 ratio). Incubation was carried out at 30 °C. For large-scale culturing, 1 l medium in a 2 l flask was inoculated with the pure culture grown on propane or butane to give an initial OD_{600} of 0·03–0·05. The flasks were made air-tight with rubber stoppers, flushed with butane (or propane when required) for 10 min and incubated on an orbital shaker (175 r.p.m.) for 48 h at 30 °C. For checking growth on other hydrocarbons (C_5–C_{10}), the cultures streaked on MMA plates were placed in a desiccator along with a glass petriplate containing a few drops of the hydrocarbon to saturate the desiccator with the vapours of the hydrocarbons. Growth of the organisms on different intermediates of alkane metabolic pathways (n-propanol, 2-propanol, tert-butanol, n-butanol, isoamyl alcohol, acetol, propanaldehyde, butyraldehyde, butyric acid, formic acid, propionic acid, pentanoic acid, hexanoic acid, capric acid and caprylic acid) was checked by streaking the culture on MMA plates containing 0·1% (v/v for liquid, w/v for solid) of different intermediates. Aldehydes were used at 0·05% (v/v) concentration. Visible growth was observed within 48–72 h on all intermediates except 2-propanol and formic acid. Growth studies were performed at 30 °C.

Membrane preparation. Late-exponential-phase cultures were harvested by centrifugation at 13000 ***g*** at 4 °C for 15 min and washed twice with PBS (10 mM phosphate buffer, 150 mM NaCl, pH 7·4). Cells from 1 l medium (about 1 g wet wt) were suspended in 1 ml 30% sucrose in PBS. DNase (25 μg ml^{-1}) and RNase (25 μg ml^{-1}) were added and mixed with 2 g glass beads (0·25–0·5 mm) (ml cell suspension)$^{-1}$. A cocktail of protease inhibitors was used throughout the preparation. The suspension was homogenized for 10 min (two 5 min cycles) at full speed in a Braun homogenizer cooled with a flow of carbon dioxide. Glass beads were removed by centrifugation at 1500 ***g*** for 10 min and the supernatant was collected. Unbroken cells and cell debris were removed by centrifugation at 10000 ***g*** for 10 min and the resulting supernatant was centrifuged at 153000 ***g*** for 2 h at 4 °C. The pellet and supernatant were collected separately. The pellet obtained from the first run was resuspended in PBS and incubated with lysozyme (100 μg ml^{-1}) for 1 h at 37 °C. After incubation the membrane fraction was purified by centrifuging twice at 153000 ***g*** and the final pellet (particulate fraction) was resuspended in 1 ml 25% (w/v) sucrose in PBS. The suspension was stored at −20 °C until further use. The supernatant was once again centrifuged at 153000 ***g*** for 2 h and the supernatant (soluble fraction) was stored at −20 °C.

Purification of hydrocarbon-induced proteins and antibody production. Electrophoresis was carried out according to the protocol of Laemmli (1970) with minor modifications. The specific bands were then cut out with a razor blade from the gel and the protein was eluted and concentrated by electrophoresis using a sample concentrator (ISCO) in Tris (25 mM)/glycine (190 mM) buffer (pH 8·3) containing 0·01% SDS for 1 h at 450 mA. The eluted protein was checked for purity on SDS-PAGE followed by silver staining. The presence of a single band on SDS-PAGE was used as a criterion of purity. Preparations showing single bands were stored at −20 °C. Antibody against this protein was raised in rabbits. Purification of IgG was carried out according to Kasper & Hartman (1987).

Construction of a genomic library in λgt11, amplification and immunoscreening. Genomic DNA of *Pseudomonas* sp. IMT40 was partially digested with *Eco*RI and 5–7 kb size fragments were purified from agarose gel using a Geneclean kit (Bio 101) according to the manufacturer's instructions. Purified DNA was ligated to λgt11 arms using the Packagene system (Promega) as per the instructions of the supplier. The phage titre was determined using *Escherichia coli* LE392 and amplification of the library was carried out following standard methods (Sambrook *et al*., 1989). Immunoscreening of the library was done with the Protoblot immunoscreening system (Promega) using *E. coli* Y1090. DNA from recombinant λ clones was isolated according to Sambrook *et al*. (1989). Recombinant λ clones were lysogenized in *E. coli* Y1089 and individual colonies were checked for their growth at 32 and 42 °C. Colonies which could not grow at 42 °C were considered lysogen. Eighty micrograms of protein from each lysogen lysate was run on SDS-PAGE according to the method of Laemmli (1970). Protein bands were visualized by silver staining (Merril *et al*., 1981). The resolved proteins were transferred onto nitrocellulose membrane (Towbin *et al*., 1979). The filters were immunodeveloped by using anti-rabbit IgG peroxidase conjugate.

Dot-ELISA. Gaseous-alkane-utilizing bacteria *Rhodococcus* sp. IMT35 and *Pseudomonas* sp. IMT40 were grown in nutrient broth, glucose and propane. Other bacteria were grown in nutrient broth, glucose and glucose in the presence of propane in gas-tight flasks. Nitrocellulose membrane was rinsed in distilled water followed by Tris-buffered saline (TBS: Tris 50 mM, pH 7·4; NaCl 150 mM) and thoroughly dried on Whatman filter paper (3 MM). Five microlitres of cell suspension was applied in duplicate and air-dried. When whole cells were applied, the dried membranes were placed in an oven at 60 °C to stabilize binding and to inactivate bacterial enzymes. Blank space on the membrane was blocked by

incubating the membrane in 3 % (w/v) casein in TBS overnight at 4 °C. The membranes were then rinsed in TBS containing 0·05 % Tween 20 and transferred in diluted antibody solution in TBS containing 1 % BSA. Diluted antibody was pre-adsorbed with *E. coli* lysate to remove non-specific IgG molecules. Excess antibodies were washed off by rinsing in TBS-Tween 20 and the membranes were incubated in alkaline-phosphatase-conjugated anti-rabbit IgG (Promega). Colour was developed using an immunoscreening kit (Promega) according to the instructions of the manufacturer.

Rapid extraction of DNA from micro-organisms and general techniques. For isolation of chromosomal DNA, different organisms were grown on LB agar media. A loopful of cells was resuspended in 50 μl TE containing 50 μg lysozyme μl^{-1} and lysed by adding 450 μl guanidinium isothiocyanate (5 M, 0·1 M EDTA). DNA was purified by chloroform/isoamyl alcohol (24:1), precipitated by 2-propanol, washed in 70 % alcohol and the dried pellet was dissolved in 100 μl water.

Ligation and restriction endonuclease digestions were done as per the instructions of the supplier (Promega) of these enzymes. DNA elution from agarose was done by using the Geneclean kit from Bio 101 and the Qiaquick gel extraction kit (Qiagen). General genetic and recombinant DNA techniques were as described by Sambrook *et al.* (1989).

Dot blotting of DNA, hybridization and autoradiography. Approximately 2 μg DNA from various organisms was applied onto Zeta probe nylon membranes using the Bio-Dot microfiltration apparatus (Bio-Rad) according to the manufacturer's instructions. Hybridization was done using different concentrations of formamide (depending on desired stringency) at 45 °C according to the instructions of the manufacturer of the nylon membranes. After hybridization, the membranes were rinsed briefly in 2 × SSC and washed in the following solutions successively: 2 × SSC + 0·1 % SDS, 0·5 × SSC + 0·1 % SDS and 0·1 × SSC + 0·1 % SDS. The membranes were dried and placed in plastic bags and exposed to X-ray film at −70 °C.

Nucleic acid labelling and purification. DNA fragments were labelled using [^{32}P]dCTP or [^{32}P]dGTP by a nick translation kit (Promega) according to the manufacturer's instructions. The labelled DNA fragments were purified using Sephadex G50 column chromatography (Sambrook *et al.*, 1989).

Construction and screening of a genomic library of *Pseudomonas* sp. IMT37. Genomic DNA from *Pseudomonas* sp. IMT37 was isolated essentially as described by Sambrook *et al.* (1989). DNA was partially digested with *Hin*dIII and ligated to the cosmid vector pHC79, also cut with *Hin*dIII and dephosphorylated. The ligated mixture was electroporated into *E. coli* MC1061.

Hybridization and screening of the genomic library. The library was screened using the 4·9 kb fragment as a probe. The 4·9 kb fragment was obtained from a genomic library of *Pseudomonas* sp. IMT40 constructed in λgt11. The immunoscreening was done with an antibody raised against a 58 kDa polypeptide which is induced by propane or butane. This DNA fragment showed high specificity of hybridization with DNAs of propane- or butane-utilizing bacteria, including *Pseudomonas* sp. IMT37, but not with non-utilizers (see Results for details). A clone, designated pRT3, with the smallest insert (6 kb) carrying the region corresponding to the encoding region of 4·9 kb was digested with *Kpn*I and the two *Hin*dIII–*Kpn*I fragments of 2·3 and 3·7 kb thus obtained were subcloned into pUC19, which was also cut with *Kpn*I and *Hin*dIII. These subclones were designated pRT3A and pRT3B. The subclone pRT3A carried the coding region for the 58 kDa protein, whereas pRT3B carried the upstream region of the ORF (Fig. 1).

Subcloning and sequencing of pRT3A and pRT3B. Overlapping subclones of pRT3A were generated in pUC19 and these were sequenced using the universal reverse and forward primers for the pUC series of plasmids. A 300 bp region of pRT3B which was upstream to the ORF in pRT3A was also subcloned in pUC19 and sequenced using the same primers. Both the strands were sequenced. A portion of the insert was also sent to Medigene (Germany) for confirmation of the sequences obtained in the laboratory. The sequence has been submitted to GenBank under accession number L81125. A homology search was carried out for the sequence using FASTA at the EMBL database, Heidelberg, Germany, and BLAST at GenBank, NCBI, NLM, Bethesda, USA.

S1 nuclease mapping and primer extension assays. Total RNA was isolated by the Qiagen RNaeasy midi kit from *Pseudomonas* sp. IMT37 grown in the presence of butane as the sole source of carbon and energy. S1 nuclease mapping was carried out as described in Sambrook *et al.* (1989). RNA (30 μg) was hybridized with the labelled probe and treated with S1 nuclease at 45 °C for 2 h. For the preparation of the probe, a plasmid, pGEMPstRT3, was constructed by cloning a ∼ 1·4 kb *Pst*I fragment of pRT3 in the *Pst*I site of pGEM5Z (Fig. 1). The construct pGEMPstRT3 was digested with *Eco*RI and the 5′ ends of digested DNA were labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase after dephosphorylating the ends with calf intestinal alkaline phosphatase. The end-labelled DNA was then digested with *Sca*I and separated in a 1 % (w/v) agarose gel. A 532 bp labelled *Eco*RI–*Sca*I fragment (Fig. 1) was cut out from gel and the DNA was eluted by a Qiaquick gel extraction kit (Qiagen).

The primer extension assay was carried out as described in Sambrook *et al.* (1989). Primer was prepared by digesting end-labelled *Eco*RI-digested pGEMPstRT3 (as described above) with *Apa*I and a 195 nt *Eco*RI–*Apa*I fragment (Fig. 1) was eluted from the gel with a Qiaquick gel extraction kit. Primer was hybridized to 30 μg IMT37 RNA for 16 h at 30 °C after initial denaturation at 85 °C for 10 min in hybridization buffer (40 mM PIPES buffer, pH 6·4; 1 mM EDTA; 0·4 M NaCl; 80 %, v/v, formamide). Reverse transcription was done at 42 °C for 1 h, the reaction was stopped by adding 1 μl 0·5 M EDTA and the remaining RNA was removed by DNase free RNaseA treatment followed by phenol/chloroform extraction. The primer extension products were precipitated with absolute ethanol at −70 °C and washed with 70 % (v/v) cold ethanol. DNA was resuspended in 8 μl TE buffer.

The extended product in the primer extension assay and the S1 nuclease protected region were analysed on a 6 % polyacrylamide sequencing gel containing 8 M urea after heating the reaction mix at 90 °C for 5 min. Samples were loaded adjacent to a DNA sequence ladder generated by using a standard primer with the single-stranded M13 bacteriophage DNA (Sequenase kit version 2.0; Amersham).

Insertional inactivation of the ORF. The longest ORF of 1512 bp in pRT3A was cut at a *Bst*EII site located at 536 bp downstream of the start codon (ATG). This was blunt-ended and ligated to the kanamycin ('kan') cassette (Pharmacia) which was also blunt-ended at the *Eco*RI ends. The plasmid carrying the kanamycin-disrupted ORF was designated pRT3AK (Fig. 1). This construct was used to transform *Pseudomonas* sp. IMT37.

Electrotransformation of *Pseudomonas*. Bacteria were grown at 37 °C until mid-exponential phase (OD_{600} 0·3) with shaking,

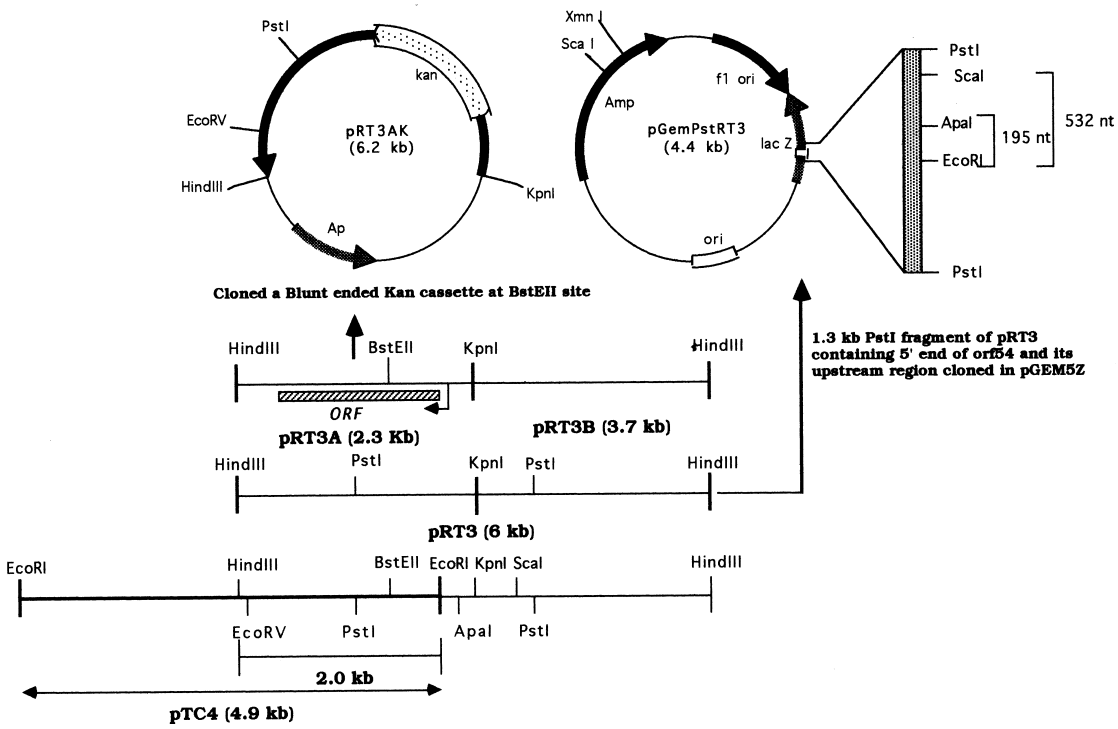

Fig. 1. Overall cloning strategy. A 4·9 kb *Eco*RI DNA fragment identified by immunoscreening using an anti-58 kDa antibody was cloned in pUC18 and the product is pTC4. The 58 kDa ORF is present in its 2·0 kb *Eco*RI–*Hin*dIII fragment and is shown as a shaded horizontal bar. A *Kpn*I–*Hin*dIII subclone (pRT3A) of pRT3 includes the above 2·0 kb *Eco*RI–*Hin*dIII fragment encoding the 58 kDa polypeptide plus 240 bases upstream of the *Eco*RI site. The direction of transcription is shown by an inverted arrow (↩) in the 2·3 kb insert of pRT3A. The ORF in pRT3A was disrupted by inserting a blunt-ended kanamycin cassette at the *Bst*EII site. This disrupted construct (pRT3AK) was used for insertional inactivation studies. A 1·4 kb *Pst*I fragment of pRT3, which contains the 5′ end and upstream region of the ORF, was cloned in pGEM5Z. A 195 nt *Apa*I–*Eco*RI and a 532 nt *Sca*I–*Eco*RI fragment from this construct (pGemPstRT3) was used in primer extension and S1 nuclease analysis, respectively. The 4·9 kb *Eco*RI fragment or a part of it, the 2·0 kb *Eco*RI–*Hin*dIII fragment, was used in probing experiments. The sizes shown in the line drawings are of the inserts only.

washed once in transformation buffer (300 mM sucrose; 7 mM sodium phosphate, pH 7·4; 1 mM $MgCl_2$) and resuspended in the same buffer to yield 10^9 c.f.u. ml^{-1} (Wirth *et al.*, 1989). Aliquots of 200 µl were made and stored at −70 °C. For electroporation, an aliquot of competent cells was thawed on ice and 100–200 ng DNA was mixed with the cells. Electroporation was done in a 0·4 cm cuvette (Bio-Rad) with the Gene Pulser (Bio-Rad) setting at 2·5 kV, 25 µF and 800 Ω. After pulsing, 800 µl LB was added to the cuvette and cells were transferred to 2 ml vials. These were incubated at 37 °C for 2 h and 100 µl of the culture was spread on appropriate plates.

Monooxygenase assay (Murrell & Ashraf, 1990). Cells were grown in MM-glucose (0·2%, w/v) for 6 h. After harvesting the cells, fresh mineral medium was added and cells were exposed to a butane/air (6:4) mixture for 10–12 h. Cells were harvested again, washed once in 20 mM Tris, pH 6·8, and resuspended in the same buffer to give a suspension of 50 mg ml^{-1}. The assay was performed in 2 ml gas chromatography vials. The assay mixture contained 50 µl cell suspension and 200 µl Tris buffer, pH 6·8 (20 mM). After equilibration for 1 h at 30 °C in a water bath, 1 ml air was drawn out and replaced with 1 ml butene (substrate) using an airtight Hamilton syringe. The vials were incubated at 37 °C for 2 h. After 2 h, 10 µl samples were removed and injected in a gas chromatograph (GC-14B; Shimadzu) fitted with a stainless steel column containing PorapakQ. The column was run isothermally at 180 °C with nitrogen (30 ml min^{-1}) as the carrier gas. The amount of epoxide was quantified from the peak area measured using a reporting integrator (Chromatopac C-R6A; Shimadzu) that was calibrated with standard solutions. Rates were expressed in nmol epoxybutane formed (g cells)$^{-1}$ h^{-1}.

RESULTS AND DISCUSSION

Isolation of gaseous-alkane-utilizing bacteria

Twenty-three colonies were isolated in pure form after repeated cycles of enrichment using LPG as carbon source. Three of them were selected for detailed studies. IMT35 was a Gram-positive coccus; IMT37 and IMT40 were Gram-negative rods. IMT40 and IMT35 could utilize both propane and butane for growth and IMT37 could grow on butane but not on propane. On the basis of morphological and biochemical characteristics, they were identified as *Rhodococcus* sp. IMT35 and *Pseudomonas* sp. IMT37 and IMT40.

Both *Rhodococcus* sp. IMT35 and *Pseudomonas* sp. IMT40 could grow well on propane, butane, pentane

and hexane but not on methane or ethane. They could utilize a wide variety of carbon sources tested (e.g. glucose, glycerol, lactate, citrate, pyruvate) and most of the intermediates (propanol, butanol, propionic acid, acetic acid, acetol) of the proposed propane and butane metabolic pathways (Woods & Murrell, 1989; Van Ginkel *et al*., 1987).

Identification and purification of a specific polypeptide induced by propane or butane and specificity of the antibody

Membrane fractions of glucose-, propane (or butane)- and nutrient-broth-grown *Pseudomonas* sp. IMT40 and *Rhodococcus* sp. IMT35 were analysed by SDS-PAGE. One unique polypeptide band of 58 kDa was apparent in propane (or butane)-grown cells, but not in glucose- or nutrient-broth-grown cells. Since the 58 kDa band was more prominent in *Rhodococcus* sp. IMT35, it was purified by electroelution. Antibody was raised against this polypeptide. Immunoblots of membrane preparations of both IMT35 and IMT40 were probed with anti-58 kDa antibody. Positive immunoreactions were obtained with the corresponding band in each only when the cells were grown on butane (Fig. 2a). Antigenically similar protein was also induced when propane was used as a growth substrate since a similar immunopositive reaction was obtained with membrane preparations of such cells (data not shown). The antibody showed no detectable reaction with membrane fractions prepared from glucose- or nutrient-broth-grown cultures (Fig. 2a). An immunoblot experiment could not detect this polypeptide in membrane preparations of cells grown on propanol or butanol, the first intermediate of the proposed propane (Woods & Murrell, 1989) and butane (Van Ginkel *et al*., 1987) pathway, respectively (data not shown). *Pseudomonas* sp. IMT37 did not appear to have an antigenically similar protein since no positive reaction could be detected against this antibody (data not shown). Hamamura *et al*. (1999) reported induction of a protein of similar molecular mass in butane-grown cells of '*P. butanovora*' and *Mycobacterium vaccae*. Based on a [^{14}C]acetylene inhibition study of butane degradation by these two bacteria, the authors suggested that this could be a component of BMO. The protein reported by them and the protein we describe here show similarities in induction and molecular mass. We have reason to believe that the 58 kDa protein we purified is also a component of BMO as described later in the paper.

The anti-58 kDa antibody was used in a dot-ELISA format against whole cells of seven micro-organisms (Table 1, marked with an asterisk) grown on propane or butane, glucose and in nutrient broth. Alkane-grown cells of IMT35 and IMT40 could be easily detected, while the same organisms grown on other substrates (glucose or nutrient broth) did not react with this antibody. Other organisms which could not utilize propane or butane failed to show any positive reactions. In order to test the sensitivity of this method, 5 µl each (in duplicate) of 10^8–10^3 propane-grown cells ml^{-1} was spotted onto a membrane filter and was processed as above. It was observed that about 5×10^3 cells per spot (i.e. 10^6 cells ml^{-1}) were required for detection by this method (data not shown). The technique of dot-ELISA gives relatively rapid results, is easy to perform and many samples can be checked on a membrane filter. Thus detection of propane- and butane-utilizing bacteria with the polyclonal antibody raised against the 58 kDa

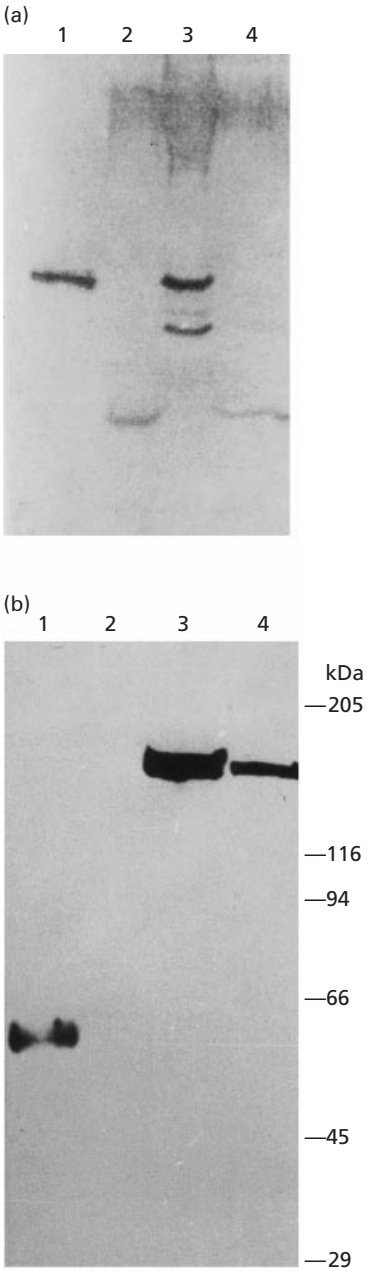

Fig. 2. (a) Western immunoblot showing the specificity of the anti-58 kDa antibody. Lanes: 1, purified 58 kDa protein; 2, 3 and 4, membrane fractions of *Rhodococcus* sp. IMT35 grown in nutrient broth, on butane and on glucose, respectively. (b) Immunoblot showing the presence of a fusion protein in crude protein extracts of recombinant lysogens. Lanes: 1, purified 58 kDa protein; 2, λgt11; 3, λTC1; 4, λTC4.

protein may open up an interesting possibility for its use in microbiological prospecting for oil and natural gas.

Analysis of a genomic library of *Pseudomonas* sp. IMT40 constructed in λgt11

Since specific antibody was available, attempts were made to find the gene which encodes this butane (or propane)-induced 58 kDa polypeptide. Immunoblot experiments showed that a similar protein was present in both *Rhodococcus* sp. and *Pseudomonas* sp. IMT40. It was therefore decided to clone the gene from the latter organism because, being a Gram-negative organism, it should be more amenable to genetic manipulation procedures. A genomic library of *Pseudomonas* sp. IMT40 was constructed in λgt11 as described in Methods. A total of nearly 10^5 plaques of an amplified genomic library were screened using anti-58 kDa antibody. Out of four putative clones, a 4·9 kb insert could be found only in two, and they were designated λTC1 and λTC4. When nick-translated 4·9 kb DNA fragments of λTC1 and λTC4 were used for hybridization under stringent conditions (50% formamide at 42 °C) with total DNA of these four clones, λTC1 and λTC4 showed a strong positive signal but no detectable reaction was obtained with λTC2 and λTC3.

These two recombinant clones containing the 4·9 kb insert were lysogenized in *E. coli* Y1089 and then crude lysates were analysed by dot-ELISA. Each of them showed the presence of a protein reacting strongly with anti-58 kDa antibody. When the lysates were run on an SDS-PAGE gel and the immunoblot was probed with anti-58 kDa antibody, both λTC1 and λTC4 lysates showed the presence of a fusion protein of about 170 kDa (Fig. 2b). Control λgt11 lysogen showed no reaction with the antibody.

The 4·9 kb inserts from λTC1 and λTC4 were re-cloned in the *Eco*RI site of pUC18 and were designated pTC1 and pTC4, respectively. Digestion of these two clones with 13 restriction endonucleases generated identical restriction patterns. Restriction endonuclease digestion of this 4·9 kb DNA fragment of *Pseudomonas* sp. IMT40 with *Hin*dIII produced a 2·9 kb and a 2·0 kb fragment (Fig. 1). These two fragments with appropriate manipulations were ligated to λgt11, packaged into lambda and then transfection was carried out. The resulting plaques were screened with anti-58 kDa antibody. DNA isolated from plaques showing positive reactions was found to contain either 2·0 kb or full 4·9 kb inserts. Plaques which showed no reaction had the 2·9 kb fragment. This implied that the 2·0 kb sub-fragment was responsible for encoding the polypeptide and the polypeptide was being expressed as a fusion protein from the 2·0 kb *Eco*RI–*Hin*dIII end.

Specificity of the 4·9 kb fragment in detection of propane/butane-utilizing bacteria

In order to check whether other bacteria had DNA sequences similar to the 4·9 kb fragment of *Pseudomonas* sp. IMT40, DNA hybridization studies were performed with DNAs of many propane/butane-utilizing as well as non-utilizing bacteria (Table 1). Genomic DNA from each bacterium was applied onto nylon membrane and probed with a ^{32}P-labelled 4·9 kb fragment at 45 °C using different concentrations (40, 45 and 50%) of formamide. It was observed that at a 40% formamide concentration, the probe yielded detectable signal with DNAs of propane- and butane-utilizing bacteria tested including the strain obtained from the NCIB, UK (*Pseudomonas* sp. MTCC 129). The other strain, *Rhodococcus* sp. MTCC 289 (a kind gift from J. J. Perry, North Carolina State University, USA), resulted in a weak but detectable signal. However, at this concentration of formamide only *Micrococcus* sp. and *Vibrio* sp., which could not utilize propane/butane, showed weak and non-specific hybridization. Such a non-specific signal was drastically reduced when the concentration of formamide was raised to 45% (Fig. 3). Among the 15 hydrocarbon-utilizing bacteria, eight

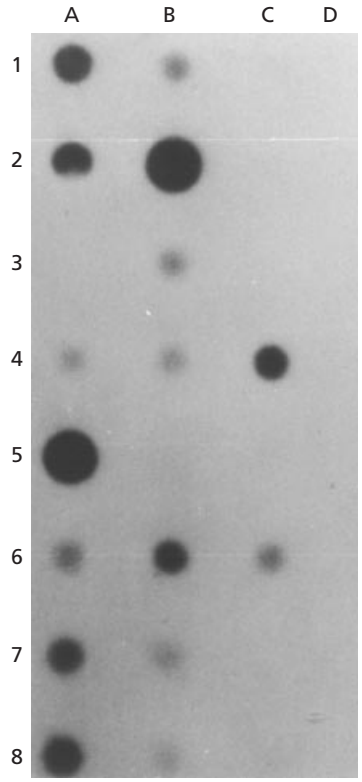

Fig. 3. Detection of propane- and butane-utilizing bacteria by a 4·9 kb DNA probe. Hybridization was carried out at 45 °C with 45% (v/v) formamide using a 4·9 kb cloned DNA fragment. Spots in the blots are genomic DNA from: A1, *Pseudomonas* sp. IMT40; A2, *Rhodococcus* sp. IMT35; A3, IMT24; A4, IMT23; A5, IMT37; A6, IMT33; A7, IMT32a; A8, IMT32b; B1, IMT39; B2, IMT41; B3, IMT14; B4, IMT34; B5, *Serratia marcescens*; B6, *Pseudomonas* sp. MTCC 129; B7, *Rhodococcus* sp. MTCC 289; B8, *Vibrio* sp.; C1, *Lactobacillus fermentum*; C2, *Gluconobacter oxydans*; C3, *E. coli*; C4, IMT21; C5, *Pseudomonas cepacia*; C6, *Micrococcus* sp.; C7, *Flavobacterium antarcticus*; C8, *Corynebacterium* sp.; D1, *Arthrobacter* sp.; D2, blank; D3, *Nocardia petroleophila*; D4, *Mycobacterium* sp.

showed a strong reaction and seven showed a weak but detectable reaction. A non-specific reaction was obtained with only one organism (*Micrococcus* sp.). At a 50% formamide concentration, the probe showed strong hybridization only with four isolates: *Pseudomonas* sp. IMT37, *Rhodococcus* sp. IMT35, IMT41 and *Pseudomonas* sp. IMT40 (from where the DNA fragment was cloned). Thus the DNA hybridization results establish that the entire 4·9 kb region is unique to bacteria which could utilize these two alkanes for growth. The DNA probe could not only detect our propane/butane-utilizing bacterial isolates, it reacted positively with two reported hydrocarbon-utilizing strains, *Pseudomonas* sp. NCIB 11309 (= MTCC 129) and *Rhodococcus* sp. MTCC 289, obtained from different geographical regions under optimized hybridization conditions (45% formamide at 45 °C).

Hybridization was also carried out with the smaller 2·0 kb *Eco*RI–*Hin*dIII region of the cloned 4·9 kb fragment as a probe and genomic DNAs of the test organisms. Reactions were performed at 45 °C in the presence of 40% formamide. The probe showed specific signals with all propane/butane-utilizing bacteria tested. Weak and non-specific hybridization was obtained with only *Micrococcus* sp. and *Corynebacterium liquefaciens* (data not shown).

The 2·0 kb *Eco*RI–*Hin*dIII fragment and 0·6 kb DNA upstream of *Eco*RI have been sequenced and analysed from *Pseudomonas* sp. IMT37 (see below). The sequence (GenBank accession no. L81125) appeared to be novel since no significant similarity was observed with available sequences in databases. The sequence of the full ORF in *Pseudomonas* sp. IMT40 DNA was also determined and was found to be identical to that of *Pseudomonas* sp. IMT37. The specificity of the DNA probes could therefore be explained on the basis of the determined base sequence, which was found to be unique to bacteria with propane/butane utilization capabilities.

Cloning and sequencing of a hydrocarbon-specific gene(s)

The conserved nature of the 4·9 kb *Eco*RI fragment among gaseous alkane utilizers suggested its importance in the pathway, but its exact role was not clear. In order to investigate the nature of the protein encoded by this fragment, we decided to compare its sequence with other known sequences. It was of interest to see if the sequence encodes a protein involved in butane utilization or is involved non-specifically in the utilization of other alkanes as well.

A genomic library of *Pseudomonas* sp. IMT37 was constructed in the *Hin*dIII site of cosmid vector pHC79 for cloning and characterizing the genes involved in the butane utilization pathway. This library was screened using the 4·9 kb fragment (described above) as a probe. The restriction map of the corresponding region in *Pseudomonas* sp. IMT37 was identical to that of IMT40. Four different types of clones having insert sizes from 6 to 26 kb were obtained. These clones covered a region of nearly 40 kb around the 4·9 kb fragment. Since it was known (described above) that a 2·0 kb *Eco*RI–*Hin*dIII region of the 4·9 kb fragment encodes the 58 kDa protein, the corresponding region from one of the clones, designated pRT3 (Fig. 1), was subcloned in pUC19 and designated pRT3A. The total insert (*Hin*dIII–*Kpn*I fragment) of 2·3 kb in pRT3A was sequenced. A 0·3 kb (*Kpn*I–*Pst*I) fragment upstream of pRT3A was also subcloned (pRT3B.1) and the sequence determined.

Sequence analysis

A search of the databases revealed no significant similarity with any known sequences, thus implying that this is a novel sequence. The entire 2606 bp sequence (GenBank accession no. L81125) was analysed using Sequaid II and MicroGenie software. The sequence was translated in all six possible frames. One ORF with two possible initiation sites (ATG) could be recognized. Irrespective of initiation at position 502 or at base 544, the termination codon (TGA) was at position 2014, thereby producing a polypeptide of 504 or 490 amino acids, respectively. Since the largest reading frame could encode a polypeptide of 54 kDa, this ORF was designated *orf54*. The molecular mass of the polypeptide in either case (54 or 52·3 kDa) is slightly less than the one (58 kDa) determined by SDS-PAGE, a phenomenon which is often reported for membrane proteins (Buchel *et al*., 1980; Youvan *et al*., 1984). Analysis of the hydropathy plot of the translated product did not reveal features indicating its possible transmembrane location except a small stretch of about 20 amino acids at the N-terminus. However, the C-terminal region was found to be rich in cysteine residues, which implies that the protein possibly has many disulfide linkages or some metal binding sites. Upstream of both the possible initiation codons, putative RBS sequences are present at bases 14 and 16 upstream of the first ATG and bases 4 and base 8 upstream of the second ATG. At present, there remains some uncertainty regarding the translational start of the protein. Six inverted repeats having free energy ranging between −12·8 and 23·0 kcal (−53·76 and 96·6 kJ) could be detected within the ORF. One of these inverted repeats (1978–2019) is present at the very end of the ORF. However, the significance of these repeats is not yet understood.

Analysis of the promoter region and the phenotypes of gene disruption mutants (see below) indicate that the gene might encode a component of multicomponent BMO. Therefore, special attention was given to compare the sequence with the known sequences of pMMO (Semrau *et al*., 1995), sMMO (Stainthorpe *et al*., 1990) and alkane monooxygenase (Kok *et al*., 1989a). No significant similarity was observed. This was not surprising because even the monooxygenases associated with hydrocarbon metabolism reported so far show very little sequence homology among themselves. The pMMO components, however, show homology with

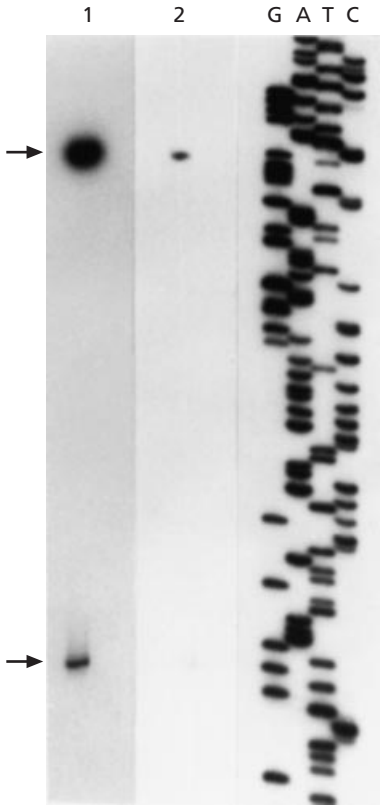

Fig. 4. Identification of the transcription start site of the gene encoding the 58 kDa protein by primer extension and S1 nuclease mapping. Arrows show the primer and the extended product in primer extension (lane 1). Lane 2 shows the protected region from the 532 nt *Eco*RI–*Sca*I fragment in S1 nuclease mapping. The sequence ladder used to determine the size was generated from single-stranded M13 bacteriophage.

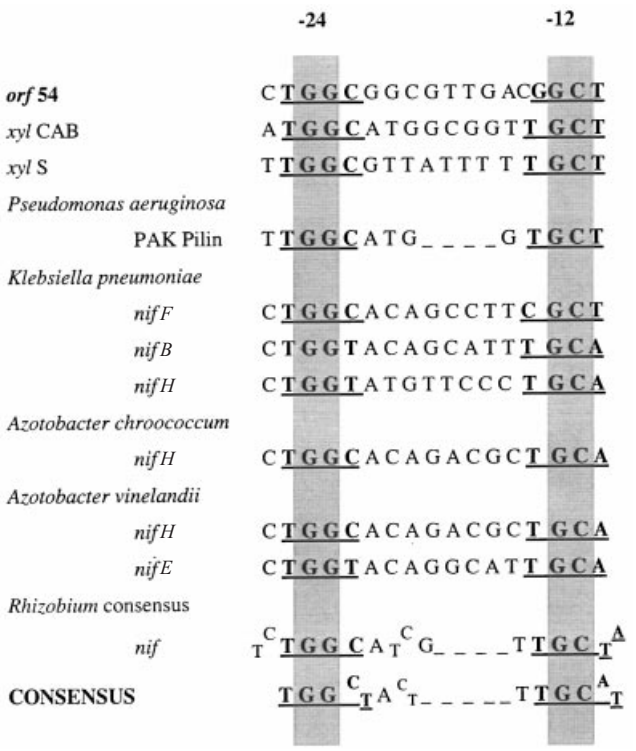

Fig. 5. Alignment of *orf54* promoter sequences with *ntr*-like promoters (Dixon, 1986; Johnson *et al.*, 1986; Deretic *et al.*, 1987). The −24 and −12 sequences are in bold and underlined and the more conserved dinucleotide sequences in these conserved sequences are shown in a shaded box.

ammonia monooxygenase (Semrau *et al.*, 1995; Holmes *et al.*, 1995).

Primer extension and S1 nuclease mapping

In order to determine the transcription start site and to identify a promoter region upstream, primer extension and S1 nuclease mapping were carried out. The size of the primer extension product was determined by comparison with the sequence ladder of pGEMPstRT3 obtained with primer ATTCCATTCTGCTGCTGCCC (PE3; bases 550–531) (data not shown) and an unrelated but known sequence ladder of M13 bacteriophage DNA. Primer extension using a 195 nt labelled *Eco*RI–*Apa*I fragment (Fig. 1) as the primer yielded a product of 267 nt and, therefore, there was an extension of 72 nt to this primer (Fig. 4). The results suggest that transcription of this ORF starts at a 'T' 261 nt upstream (5′) of the start codon ATG, located at 544.

S1 nuclease mapping, done with a 532 nt labelled (*Eco*RI–*Sca*I) fragment (Fig. 1), also showed a protected region which moves in the sequencing gel at a position which was identical to that of the primer extension product and is therefore 267 nt long (Fig. 4). Thus, similar results obtained from both the primer extension assay as well as S1 nuclease mapping show convincingly that transcription starts at base 283. *Pseudomonas* is known to possess *E. coli* −35 and −10 consensus sequence as well as its unique promoter sequence (TGGC at the −24 and TGCT at the −12 position; Deretic *et al.*, 1987). In this ORF, the promoter sequence is similar to *Pseudomonas* and has an identical TGGC at the −24 position and GGCT at −11. The conclusion that transcription starts at base 283 is also reinforced by the presence of a promoter at an ideal distance upstream.

The position and the sequence of the promoter bear close resemblance to promoters in other systems such as *xylA* in *Pseudomonas putida*, several *nif* genes in *Klebsiella pneumoniae*, *Azotobacter chroococcum* and *Azotobacter vinelandii* (Dixon, 1986) and also the pilin gene in *Pseudomonas aeruginosa* (Johnson *et al.*, 1986). Common features of these genes are: (a) their transcription is *ntrA*-dependent (Dixon, 1986), (b) involvement of an activator protein (Kustu *et al.*, 1989) and (c) a characteristic conserved GC doublet at around −12 bp and a conserved GG doublet at around −24 bp upstream of the transcription start site. An alignment of the *orf54* promoter sequence with a few other *ntrA*-dependent genes is shown (Fig. 5). It is very likely, therefore, that this gene encodes an enzymic rather than

a regulatory protein and may also need the *ntrA* product for its transcription.

Insertional inactivation of the ORF

In order to ascertain the function of the 1512 bp ORF from pRT3A in hydrocarbon utilization, it was disrupted at 536 bp downstream of the ATG start codon in the ORF using a kanamycin cassette. The ORF in pRT3A was digested with *Bst*EII and blunt-ended. A kanamycin cassette (1·3 kb), having *Eco*RI ends (Pharmacia), was also blunt-ended with Klenow and ligated to pRT3A. The recombinant plasmid, designated pRT3AK, was electroporated into *Pseudomonas* sp. IMT37. A total of 43 kanamycin-resistant transformants were selected for preliminary characterization. These transformants were initially checked for their ability to grow on butane, pentane and hexane. Five out of 43 transformants were unable to utilize any one of these alkanes and have a possible disruption in the target gene. These were designated 37.1K, 37.2K, 37.3K, 37.4K and 37.5K. Three kanamycin-resistant transformants which could grow on these hydrocarbons were also selected for analysis. These were designated 37.6K, 37.7K and 37.8K.

Since pUC19-based plasmids could not survive in *Pseudomonas* and since the spontaneous frequency of kanamycin resistance was below a detectable level, the only way these transformants could become kanamycin resistant was by integration of the plasmid (pRT3AK) into the chromosome by homologous recombination. Homologous recombination may result from single or double crossover events. Southern hybridization of chromosomal DNA isolated from the six selected kanamycin-resistant mutants confirmed the presence of the kanamycin cassette (data not shown). When genomic DNA was digested with *Eco*RI and probed with the labelled 4·9 kb fragment, wild-type *Pseudomonas* sp. IMT37 revealed, as expected, only one band of 4·9 kb whereas the mutant 37.3K showed a band of 6·2 kb (data not shown). The increased size of this fragment was due to a double crossover event between the insert (having the ORF disrupted by a kanamycin cassette) and the chromosomal DNA. Four other mutants (37.1K, 37.4K, 37.7K and 37.8K) showed two bands of 4·9 kb and 6·2 kb on hybridization with the 4·9 kb fragment. This observation is in agreement with integration of the plasmid pRT3AK by a single homologous crossover event. Single crossover would result in integration of the whole plasmid and this event would still retain an undisrupted copy of the gene while the other one will be disrupted. Such kanamycin-resistant transformants, therefore, should not lose the ability to grow on propane and butane. The majority of the transformants, exemplified by 37.6K, 37.7K and 37.8K, belong to this group as expected. In spite of originating from single crossover events, as revealed by Southern analysis, four (37.1K, 37.2K, 37.4K and 37.5K) out of 43 transformants could not utilize any of the alkanes tested. Their phenotype appears to be similar to the transformant 37.3K, which represents a double crossover phenomenon. This unexpected behaviour could be attributed to the formation of an incorrect reading frame at the point of single crossover. Similar observations were also reported by Martin & Murrell (1995).

Growth of all these transformants was also checked on other hydrocarbons and on different intermediates of their metabolic pathways as sole carbon sources. Five transformants (37.1K–37.5K) did not grow on any of the hydrocarbons tested viz. butane, pentane, hexane, heptane, octane, nonane and decane, but on the other hand they were able to grow on n-butanol, 1-propanol, hexanoic acid and caprylic acid. Three transformants, 37.6K, 37.7K and 37.8K, and the wild-type *Pseudomonas* sp. IMT37 were able to grow on all these carbon sources. The inability of these transformants to grow on butane was also confirmed using liquid minimal medium (MM), with butane as the sole carbon source. This inability of mutants to utilize butane might be due to the loss of monooxygenase activity that converts hydrocarbons to their respective alcohols. In order to check that the disruption of the ORF in pRT3A results in the loss of monooxygenase activity, a whole cell enzyme assay was performed. Monooxygenase activity in one of the mutants, 37.1K (unable to grow on hydrocarbons), was not detectable. In another transformant, 37.7K (kanamycin-resistant but able to grow on hydrocarbons), the monooxygenase activity [131·8 nmol h^{-1} (g cell wet wt)$^{-1}$] was comparable to the wild-type activity [134 nmol h^{-1} (g cells)$^{-1}$]. This observation suggests that in these five mutants (37.1AK–37.5AK) the defect is in the first step of the metabolic pathway and not in any subsequent step because they can utilize intermediates such as alcohols and acids. This implies that alcohol dehydrogenase, aldehyde dehydrogenase and other enzymes involved in the pathway are not affected. The phenotype of the mutants and analysis of the sequence indicate that the ORF encodes an essential component of BMO and not a regulatory protein. Further experimentation will be necessary to confirm this hypothesis. As is evident from the growth studies, inactivation of the gene results in the loss of the ability to utilize a series of hydrocarbons from C_4 to C_{10}. The result therefore shows that functional integrity of the gene is essential for utilization of alkanes as carbon and energy source. It may not be possible, at present, to conclude that this organism uses the same genetic and metabolic route for utilization of the alkanes tested but the results definitely support the view that the product of this ORF is involved in catalysing the conversion of at least seven alkanes (C_4–C_{10}) to the respective alcohols. The protein encoded by the gene therefore appears to show broad specificity in its action towards alkanes from C_4 to C_{10}.

Although there exists some physiological evidence to substantiate the pathway proposed for butane metabolism in bacteria (van Ginkel *et al.*, 1987; Arp, 1999), no information is available about the nature of the proteins involved and the genetic machinery they employ. We were able to isolate for the first time a polypeptide specifically induced by butane and clone and sequence the DNA fragment encoding this protein. The specificity

of the anti-58 kDa antibody for the detection of bacteria which were actively utilizing a gaseous alkane(s) could be explained on the basis of the polypeptide being induced specifically by such a substrate(s). Since the DNA sequence encoding this polypeptide was found to be novel, we could show that the DNA fragment could be used as a probe for detection of such microbes. By a marker exchange mutagenesis approach, it was possible to identify and characterize for the first time a unique gene induced by butane. The organism could grow on other higher linear alkanes; however, following the disruption of this gene its ability to utilize other hydrocarbons of length C_4–C_{10} is abolished. It seems, therefore, that the same gene may also be induced by other alkanes (C_5–C_{10}) and the gene product is essential for metabolism of these hydrocarbons. This implies a broad specificity of the system. On the other hand, the organism can not utilize alkanes shorter than butane. Therefore, a mechanism probably exists to measure the chain length of hydrocarbons that the organism encounters. This possibility needs to be confirmed by further work.

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REFERENCES

Arp, D. J. (1999). Butane metabolism by butane grown '*Pseudomonas butanovora*'. *Microbiology* **145**, 1173–1180.

Buchel, D. E., Gronenborn, B. & Müller-Hill, B. (1980). Sequence of the lactose permease gene. *Nature* **283**, 541–545.

Colby, J., Stirling, D. I. & Dalton, H. (1977). The soluble methane monooxygenase of *Methylococcus capsulatus* (Bath). Its ability to oxygenate *n*-alkanes, *n*-alkenes, ethers and alicyclic, aromatic and heterocyclic compounds. *Biochem J* **165**, 395–402.

Colby, J., Dalton, H. & Whittenbury, R. (1979). Biological and biochemical aspects of microbial growth on C1 compounds. *Annu Rev Microbiol* **33**, 481–517.

Coleman, J. P. & Perry, J. J. (1985). Purification and characterization of the secondary alcohol dehydrogenase from propane utilizing *Mycobacterium vaccae* JOB 5. *J Gen Microbiol* **131**, 2901–2907.

Dalton, H. (1980). Oxidation of hydrocarbons by methane monooxygenase from a variety of microbes. *Adv Appl Microbiol* **131**, 2901–2907.

Dalton, H. S., Prior, D., Leak, D. J. & Stanley, S. H. (1984). Regulation and control of methane monooxygenase. In *Microbial Growth on C1 Compounds*, pp. 75–82. *Proceedings of the 4th International Symposium*. Edited by R. L. Crawford & R. S. Hanson. Washington, DC: American Society for Microbiology.

Deretic, V., Gill, J. F. & Chakrabarty, A. M. (1987). Alginate biosynthesis: a model system for gene regulation and function in *Pseudomonas*. *Biotechnology* **5**, 469–477.

Dixon, R. (1986). The *xylABC* promoter from the *Pseudomonas putida* TOL plasmid is activated by nitrogen regulatory genes in *Escherichia coli*. *Mol Gen Genet* **203**, 129–136.

Eggink, G., Lageveen, R. C., Attenburg, B. & Witholt, B. (1987a). Controlled and functional expression of the *Pseudomonas oleovorans* alkane utilizing system in *Pseudomonas putida* and *Escherichia coli*. *J Biol Chem* **262**, 17712–17718.

Eggink, G., van Lelyveld, P. H., Arnberg, A., Arfman, N., Witteveenv, C. & Witholt, B. (1987b). Structure of the *Pseudomonas putida alk*BAC operon. *J Biol Chem* **262**, 6400–6406.

Eggink, G., Engel, H., Meijer, W. G., Otten, J., Kingma, J. & Witholt, B. (1988). Alkane utilization in *Pseudomonas oleovorans*: structure and function of the regulatory locus *alk*R. *J Biol Chem* **263**, 13400–13405.

Eggink, G., Engel, H., Vriend, G., Terpstra, P. & Witholt, B. (1990). Rubredoxin reductase of *Pseudomonas oleovorans*: structural relationship to other flavoprotein oxidoreductases based on one NAD and two FAD finger prints. *J Mol Biol* **212**, 135–142.

Fox, B. G. & Lipscomb, J. D. (1988). Purification of a high specific activity methane monooxygenase hydroxylase component from a type II methanotroph. *Biochem Biophys Res Commun* **154**, 165–170.

Fox, B. G., Surerus, K. K., Munck, E. & Lipscomb, J. D. (1988). Evidence for a μ-oxo-bridged binuclear iron-cluster in the hydroxylase component of methane monooxygenase, Mossbauer and EPR studies. *J Biol Chem* **263**, 10553–10556.

Fox, B. G., Froland, W. A., Degde, J. E. & Lipscomb, J. D. (1989). Methane mono-oxygenase from *Methylosinus trichosporium* OB3b. *J Biol Chem* **264**, 10023–10033.

Green, J. & Dalton, H. (1989). A stopped flow kinetic study of soluble methane monooxygenase from *Methylococcus capsulatus* (Bath). *Eur J Biochem* **153**, 137–144.

Grunstein, M. & Hogness, D. S. (1975). Colony hybridization, a method for the isolation of cloned DNAs that contain a specific gene. *Proc Natl Acad Sci U S A* **72**, 3961–3965.

Hamamura, N., Storfa, R. T., Semprini, L. & Arp, D. J. (1999). Diversity in butane monooxygenases among butane-grown bacteria. *Appl Environ Microbiol* **65**, 4586–4593.

Hanson, R. S. (1980). Ecology and diversity of methylotrophic organisms. *Adv Appl Microbiol* **26**, 3–39.

Higgins, I. J. (1980). Respiration in methylotrophic bacteria. In *Diversity of Bacterial Respiratory Systems*, pp. 187–221. Edited by C. J. Knowles. Boca Raton, FL: CRC Press.

Hohn, B. & Collins, J. (1980). A small cosmid for efficient cloning of large DNA fragments. *Gene* **11**, 291–298.

Holben, W. E., Jansson, J. E., Chelm, B. K. & Tiedje, J. M. (1988). DNA probe method for the detection of the soil bacterial community. *Appl Environ Microbiol* **54**, 703–711.

Holmes, A. J., Costello, A., Lidstrom, M. E. & Murrell, J. C. (1995). Evidence that particulate methane monooxygenase and ammonia monooxygenase may be evolutionarily related. *FEMS Microbiol Lett* **132**, 203–208.

Johnson, K., Parker, M. L. & Lory, S. (1986). Nucleotide sequence and transcriptional initiation site of two *Pseudomonas aeruginosa* pilin genes. *J Biol Chem* **261**, 15703–15708.

Kasper, C. W. & Hartman, P. A. (1987). Production and specificity of monoclonal antibodies and polyclonal antibodies to *Escherichia coli*. *J Appl Bacteriol* **63**, 335–341.

Kok, M., Oldenhuis, R., Van der Linden, M. P. G., Raatjes, P., Kingna, J., Van Lelyveld, P. H. & Witholt, B. (1989a). The *Pseudomonas oleovorans* alkane hydroxylase gene: sequence and expression. *J Biol Chem* **264**, 5435–5441.

Kok, M., Oldenhuis, R., Van der Linden, M. P. G., Meulenberg, C. H. C., Kingna, J. & Witholt, B. (1989b). The *Pseudomonas oleovorans alk*BAC operon encodes two structurally related rubredoxins and an aldehyde dehydrogenase. *J Biol Chem* **264**, 5442–5451.

Kustu, S., Santero, E., Keener, J., Popham, D. & Weiss, D. (1989). Expression of sigma 54 (ntrA)-dependent genes is probably united by a common mechanism. *Microbiol Rev* **53**, 367–376.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lidstrom, M. E. & Sterling, D. T. (1990). Methylotrophs: genetics and commercial applications. *Annu Rev Microbiol* **44**, 27–58.

Lonsane, B. K., Singh, H. D. & Baruah, J. N. (1977). Geomicrobiological methods for prospecting of petroleum oil gases – a neglected aspect of petroleum prospecting. *J Sci Ind Res* **36**, 534–541.

Lukins, H. B. & Foster, J. W. (1963). Methylketone metabolism by hydrocarbon utilizing *Mycobacteria*. *J Bacteriol* **85**, 1074–1087.

Martin, H. & Murrell, J. C. (1995). Methane monooxygenase mutants of *Methylosinus trichosporium* constructed by marker exchange mutagenesis. *FEMS Microbiol Lett* **127**, 243–248.

Merril, C. R., Goldman, D., Sedman, S. A. & Ebert, M. H. (1981). Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science* **211**, 1437–1438.

Miyoshi, M. (1895). Die Durchbohrung von Membranen durch Pilzfaden. *J Wiss Biot* **28**, 269–289. [Cited in *Petroleum Microbiology*. Edited by R. M. Atlas. New York: Macmillan.]

Murrell, J. C. & Ashraf, W. (1990). Cell free assay methods for enzymes of propane utilization. *Methods Enzymol* **188**, 26–32.

Ogram, A. V., Sayler, G. S. & Barkay, T. (1987). The extraction and purification of microbial DNA from sediments. *J Microbiol Methods* **7**, 57–66.

Quayle, J. R. (1980). Aspects of the regulation of methylotrophic metabolism. *FEBS Lett* **117**, K16–K27.

Quayle, J. R. & Ferenci, T. (1978). Evolutionary aspects of autotrophy. *Microbiol Rev* **42**, 251–273.

Rosenzweig, A. C., Frederick, C. A., Lippard, S. J. & Nordlund, P. (1993). Crystal structure of a bacterial non-haem iron hydroxylase that catalyses the biological oxidation of methane. *Nature* **366**, 537–543.

Saeki, H. & Furahashi, K. (1994). Cloning and characterization of a *Nocardia corallina* B-276 gene cluster encoding alkene monooxygenase. *J Ferment Bioeng* **78**, 399–404.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sayler, G. S., Shieldo, M. S., Tedford, E., Breen, A., Hooper, S., Sirotkin, K. & Davis, J. (1985). Application of DNA-DNA colony hybridization for the detection of catabolic genotypes in environmental samples. *Appl Environ Microbiol* **49**, 1295–1303.

Sealy, J. Q. (1974). A geomicrobiological method of prospecting for petroleum. *Oil Gas J* **72**, 98.

Semrau, J. D., Chistoserdov, A., Lebron, J. & 7 other authors (1995). Particulate methane monooxygenase genes in methanotrophs. *J Bacteriol* **177**, 3071–3079.

Söhngen, N. L. (1906). Über Bakterien, Welche Methan als Kohlenstoffnahrung Energiquelle Gebrauchen. *Zentbl Bakteriol Parasitenkd Abt II* **15**, 513–517. [Cited in *Petroleum Microbiology*. Edited by R. M. Atlas. New York: Macmillan.]

Stainthorpe, A. C., Lees, V., Salmond, G. P. C., Dalton, H. & Murrell, J. C. (1990). The methane monooxygenase gene cluster of *Methylococcus capsulatus* (Bath). *Gene* **91**, 27–34.

Taggart, M. S. (1967). Petroleum prospecting. In *Petroleum Microbiology*, pp. 195–245. Edited by J. B. Davis. Amsterdam: Elsevier.

Torsvik, V. L. (1980). Isolation of bacterial DNA from soil. *Soil Biol Biochem* **12**, 15–21.

Towbin, H., Staehelin, T. & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* **76**, 4350–4354.

Van Ginkel, C. G., Welten, H. G. J., Hartmans, S. & de Bont, J. A. M. (1987). Metabolism of trans-2-butene and butane in *Nocardia* TB1. *J Gen Microbiol* **133**, 1713–1720.

Whittenbury, R., Phillips, K. C. & Wilkinson, J. F. (1970). Enrichment, isolation and some properties of methane utilizing bacteria. *J Gen Microbiol* **61**, 205–218.

Wirth, R., Friesenegger, A. & Fiedler, S. (1989). Transformation of various species of gram negative bacteria belonging to 11 different genera by electroporation. *Mol Gen Genet* **216**, 175–177.

Woodland, M. P. & Dalton, H. (1984). Purification and characterization of component A of the methane monooxygenase from *Methylococcus capsulatus* (Bath). *J Biol Chem* **259**, 53–59.

Woods, N. R. & Murrell, J. C. (1989). The metabolism of propane in *Rhodococcus rhodochrous* PNKb1. *J Gen Microbiol* **135**, 2335–2344.

Yanisch-Perron, C., Vieira, J. & Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.

Youvan, D. C., Bylina, E. J., Alberti, M., Begusch, H. & Hearst, J. E. (1984). Nucleotide and deduced polypeptide sequences of the photosynthetic reaction center, B870 antenna and flanking polypeptides from *R. capsulata*. *Cell* **37**, 949–957.

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