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Molecular analysis of the soluble butane monooxygenase from '*Pseudomonas butanovora*'

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'*Pseudomonas butanovora*' is capable of growth with butane via the oxidation of butane to 1-butanol, which is catalysed by a soluble butane monooxygenase (sBMO). *In vitro* oxidation of ethylene (an alternative substrate for sBMO) was reconstituted in the soluble portion of cell extracts and was NADH-dependent. Butane monooxygenase was separated into three components which were obligately required for substrate oxidation. The N-terminal sequences of the peptides associated with butane monooxygenase led to the cloning and sequencing of the 5797 nucleotide *bmo* gene cluster. Comparisons of the deduced amino acid sequences with other multicomponent monooxygenases suggest that sBMO is a multimeric hydroxylase with 61, 45 and 19 kDa subunits encoded by *bmoXYZ*, a 40 kDa oxidoreductase encoded by *bmoC*, and a 15 kDa regulatory protein encoded by *bmoB*. A sixth structural gene (*bmoD*) encodes a 9·6 kDa protein with similarity exclusively to *mmoD* (*orfY*), a putative metal centre assembly protein of the soluble methane monooxygenases. Insertional inactivation of *bmoX* resulted in a mutant '*P. butanovora*' strain incapable of growth with butane. A putative promoter element characteristic of promoters associated with σ^{54}-dependent transcription initiation was located upstream of the *bmo* genes. Expression of all six genes was detected in butane-induced cells. Butane monooxygenase from '*P. butanovora*' aligns most closely with non-haem carboxylate-bridged diiron monooxygenases and, moreover, contains the characteristic iron-binding motif. The structural and mechanistic implications of the high sequence identity (up to 64%) between the peptides of butane monooxygenase and methane monooxygenases are discussed.

Keywords: butane metabolism, alkane oxidation, diiron monooxygenase

INTRODUCTION

'*Pseudomonas butanovora*' is capable of growth with alkanes (C_2–C_9), alcohols (C_2–C_4) and organic acids as sources of carbon and energy (Takahashi *et al.*, 1980). The 16S rRNA analysis suggests that the Gram-negative '*P. butanovora*' is a β-proteobacterium of the *Rhodocyclus* group, related to members of the genus *Thauera* (Anzai *et al.*, 2000). Butane metabolism in '*P. butanovora*' proceeds by terminal hydroxylation of butane to 1-butanol. Subsequent oxidation reactions are first catalysed by butanol dehydrogenases to form butyraldehyde (Vangnai *et al.*, 2002), followed by oxidation of butyraldehyde to form butyrate (Arp, 1999), and probably metabolized further through the β-oxidation pathway of fatty acid metabolism. The two 1-butanol dehydrogenases induced in butane-grown cells have been characterized biochemically and at the molecular level (Vangnai & Arp, 2001; Vangnai *et al.*, 2002). Much less is known about the first enzyme in the catabolic pathway, butane monooxygenase. The butane monooxygenase of '*P. butanovora*' has also been implicated

Abbreviations: BMOB, butane monooxygenase regulatory protein; BMOD, *bmoD* gene product; BMOH, butane monooxygenase hydroxylase; BMOR, butane monooxygenase reductase; MMOB, methane monooxygenase regulatory protein; MMOD, *mmoD* (*orfY*) gene product; MMOH, methane monooxygenase hydroxylase; MMOR, methane monooxygenase reductase; pMMO, particulate methane monooxygenase; sBMO, soluble butane monooxygenase; sMMO, soluble methane monooxygenase.

The GenBank accession number for the *bmoXYBZDC* sequence is AY093933.

in the co-oxidation of chlorinated hydrocarbons and thus has potential use for bioremediation (Hamamura *et al.*, 1997).

Non-specific bacterial oxygenases used for bacterial growth with aliphatic or aromatic compounds employ an array of prosthetic groups including flavin, haem, copper, binuclear iron centres and mononuclear iron centres (Arp *et al.*, 2001). Propane-oxidizing bacteria which utilize terminal and subterminal oxidation pathways have been identified (Ashraf *et al.*, 1994; Stephens & Dalton, 1986), but the propane monooxygenases have not been further characterized. Bacteria capable of metabolizing liquid alkanes do so by a terminal oxidation pathway and utilize three-component systems containing an integral membrane alkane hydroxylase, rubredoxin and rubredoxin reductase. Examples include the alkane hydroxylases from *Pseudomonas oleovorans* (McKenna & Coon, 1970) and *Acinetobacter* ADP1 (Ratajczak *et al.*, 1998), xylene monooxygenase (Suzuki *et al.*, 1991) and membrane desaturases, which belong to a distinct category within the diiron monooxygenase family (Shanklin *et al.*, 1994). Methane-oxidizing bacteria possess a particulate methane monooxygenase (pMMO), which may contain copper at the active site (Zahn & DiSpirito, 1996). Under conditions of copper starvation, some methanotrophs can use a soluble diiron methane monooxygenase (sMMO) with different protein composition and iron coordination than alkane hydroxylases (Stanley *et al.*, 1983). The sMMO enzymes are three-component systems consisting of a soluble hydroxylase, an NADH-oxidoreductase and a small regulatory protein (Colby & Dalton, 1978; Fox *et al.*, 1989). The soluble methane monooxygenase hydroxylase from methanotrophic bacteria is a prototypical non-haem carboxylate-bridged diiron monooxygenase that has been characterized extensively (Lipscomb, 1994; Wallar & Lipscomb, 1996). The presence of a μ-hydroxo-bridged iron centre was determined by X-ray crystallography and spectroscopy (Dewitt *et al.*, 1991; Elango *et al.*, 1997; Rosenzweig *et al.*, 1993). The substrate range of sMMO includes C_1–C_7 alkanes, alkenes and some aromatic compounds (Green & Dalton, 1989). Soluble toluene monooxygenases have also been described, including four-component diiron monooxygenases (Pikus *et al.*, 1996), three-component diiron monooxygenases (Newman & Wackett, 1995) and three-component mononuclear iron dioxygenases (Subramanian *et al.*, 1979, 1981, 1985).

The diversity among bacterial enzymes that degrade aliphatic and aromatic compounds is also found among those that initiate butane metabolism (Hamamura *et al.*, 1999). *Nocardioides* sp. strain CF8 utilizes a copper-containing enzyme reminiscent of pMMO and ammonia monooxygenase, while '*P. butanovora*' utilizes an uncharacterized monooxygenase that does not require copper (Hamamura *et al.*, 1999). Biochemical and genetic information about enzymes that catalyse the oxidation of short-chain, gaseous alkanes is largely absent. Recently, a gene encoding a 54 kDa polypeptide was shown to be involved in butane oxidation in *Pseudomonas* sp. IMT37. The sequence was not similar either to alkane hydroxylases (liquid alkanes) or to methane monooxygenase, and furthermore did not have significant similarity to any entries in the protein sequence databases (Padda *et al.*, 2001). This work describes the butane monooxygenase genes from '*P. butanovora*' and confirms that the cluster is involved in butane metabolism and is similar to *mmo* gene clusters.

METHODS

Reagents. All chemicals used in this study were of analytical grade. Butane gas (99 %) was purchased from Airgas. Acetylene was generated by the addition of H_2O to CaC_2.

Cell culture and preparation of cell extracts. '*Pseudomonas butanovora*' was cultured at 30 °C in sealed serum bottles as previously described (Hamamura *et al.*, 1997) except that yeast extract and CO_2 were omitted. The carbon source supplied for growth was either 7 % (v/v) butane added as an overpressure, 10 mM sodium lactate or 10 mM sodium citrate. Cells were typically harvested between 0·5 and 0·7 OD_{600}. '*P. butanovora*' was also cultured in a sealed 17 litre fermenter sparged with 7 % butane (v/v) and periodic replenishments of O_2 to 20 % (v/v). An average of 2 g of cells (wet weight) per litre of culture was obtained. Cells were harvested by centrifugation (10 min at 5000 ***g***; 10 °C), washed by resuspension with 30 mM potassium phosphate (pH 7·2), pelleted and stored as a paste at −70 °C. Frozen cells were thawed in 3 vols lysis buffer per mg cell paste. The lysis buffer consisted of buffer A [25 mM MOPS (pH 7·2), 5 % (v/v) glycerol, 1 mM DTT], plus 200 μM $Fe(NH_4)_2(SO_4)_2$, 2 mM cysteine, 0·1 mg deoxyribonuclease I ml^{-1} (Sigma) and 0·2 mg lysozyme ml^{-1}. After three freeze–thaw cycles, the lysate was subjected to sonication with a microtip (Ultrasonics) for four cycles of 30 s pulses. The cell extract was clarified by ultracentrifugation (105 000 ***g*** for 1 h).

Assay conditions. Whole-cell assays were performed in 10 ml sealed serum vials using ethylene, an alternative substrate for butane monooxygenase (Sayavedra-Soto *et al.*, 2001). Cells (0·5 mg protein) were incubated at 30 °C in 1 ml buffer [30 mM potassium phosphate (pH 7·2), 2 mM sodium butyrate] and with 20 % (v/v) ethylene in the head-space. Formation of ethylene oxide was detected by gas chromatography as described by Hamamura *et al.* (1999). *In vitro* assays contained 25 mM MOPS (pH 7·2), 2 mM NADH, 20 % (v/v) ethylene, and 0·1–2·0 mg cell extract or column fractions. Acetylene, a mechanism-based inactivator of butane monooxygenase (Hamamura *et al.*, 1999), was used to inactivate the substrate-binding component of butane monooxygenase. Cell suspensions were incubated as above, but with acetylene [1 % (v/v)] replacing ethylene. After incubation for 30 min at 30 °C with shaking, the cells were washed, lysed, and the resulting cell extract was clarified by ultracentrifugation as above. This 'acetylene-inactivated' extract (6·5 mg protein) was used as a source of acetylene-insensitive soluble butane monooxygenase (sBMO) components in the activity assays where indicated.

Separation of the butane monooxygenase components. The soluble portion of the cell extract was applied to a 1·6 × 18 cm Q-Sepharose FF column equilibrated with buffer A (see above). The column was washed with 70 ml buffer A and developed with a 350 ml linear gradient from 0 to 550 mM NaCl in buffer A. The first component, proposed to be the regulatory protein, eluted between 250 and 300 mM NaCl. Individual fractions that were able to reconstitute butane

Table 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant properties	Reference or source
'Pseudomonas butanovora'		
ATCC 43655	Wild-type	ATCC
Pbu41	ATCC 43655 derivative, *bmoX*::*lacZ*::kan^r cassette inserted at *Bam*HI site (position 259)	This study
Escherichia coli		
JM109	*endA1 recA1 gyrA96 thi hsdR17* (r_k^- m_k^-) *relA1 supE44* Δ(*lac–proAB*) [F′ *traD36 proAB lacI*^{q}*Z*ΔM15]	Promega (Yanisch-Perron *et al*., 1985)
LE392	*hsdR514* (r_k^- m_k^-) *supE44 supF58 lacY1 galK2 galT22 metB1 trpR55*	Murray *et al*. (1977)
Plasmids and λ clones		
λPbu17	λ clone containing partial *bmoX* and complete *bmoYBZ* (2·9 kbp)	This study
pPbu281	PCR product (3·4 kbp) with introduced flanking *Xba*I sites containing complete *bmoX* and partial *bmoY*; cloned in the *Xba*I site of pBluescript II SK(−)	This study
pPbu2	pPbu281 subclone	This study
pPbu41	pPbu2 subclone with a *lacZ*::kan^r cassette inserted at *bmoX Bam*HI site (position 259)	This study
Cloning vectors		
λ Gem11	λ vector used to construct the genomic library of '*P. butanovora*'	Promega
pBluescript II SK(−)	2961 bp phagemid derived from pUC19; multiple cloning cassette; Amp^r	Stratagene; GenBank no. X52330
pGEM-T Easy	PCR product cloning vector; multiple cloning cassette includes flanking *Eco*RI sites; Amp^r	Promega

monooxygenase activity by complementing the hydroxylase and reductase components were pooled. The second component, proposed to be the hydroxylase component, eluted between 320 and 360 mM NaCl and the third component, proposed to be the reductase component, eluted between 400 and 450 mM NaCl. Likewise, the individual fractions which complemented the other two protein components to reconstitute butane monooxygenase activity were pooled and concentrated by ultrafiltration (Millipore; YM100 for the hydroxylase component, YM10 for the reductase component).

Protein characterizations. SDS-PAGE (10% total gel, 2·6% cross-linker running gel) was performed following the Laemmli procedure (Laemmli, 1970) and the proteins were stained with Coomassie blue. The apparent molecular masses of polypeptides were determined by comparison with R_f values of standard proteins. The partially purified components associated with butane monooxygenase activity were resolved by SDS-PAGE and electroblotted onto PVDF membranes (Immobilon-P; Millipore). The N-terminal amino acid sequences of the polypeptides were determined by Edman degradation at the Institute of Molecular Biology Biotechnology Laboratory, University of Oregon. Protein concentrations were determined by the biuret assay (Gornall *et al*., 1949). Whole cells were first solubilized with 3 M NaOH for 30 min at 65 °C. Bovine serum albumin was used as the standard.

Plasmids, bacterial strains and nucleic acid manipulations. Table 1 summarizes the plasmids and strains used in this study. DNA manipulations were performed using standard protocols (Sambrook *et al*., 1989). Degenerate primers for PCR DNA amplification were designed from the N-terminal amino acid sequences of the butane monooxygenase polypeptides with the consensus-degenerate hybrid oligonucleotide primer strategy (CODEHOP; Rose *et al*., 1998). The forward primer sequence was 5′-ATCTTCACCCGCGGCATGGTN-GAYCCNGA-3′ and the reverse primer sequence was 5′-GGCGACGATCTTGTACTGYTGCATNARCAT-3′. DNA was amplified by PCR using *Taq* DNA polymerase (Fermentas) following the directions of the manufacturer. The amplified DNA product was cloned into pGEM-T Easy (Promega) using *Escherichia coli* strain JM109 as host and used to screen the genomic library. The genomic library of '*P. butanovora*' was constructed in λ-Gem11 (Promega) with *E. coli* strain LE392 as host (Sambrook *et al*., 1989). To sequence the portions of the *bmo* genes not represented by the library clone, genomic DNA was digested with either *Eco*RI or *Bam*HI, religated, and used as a template for inverse PCR (Sambrook *et al*., 1989). These inverse PCR amplification products were cloned, sequenced, and used to design primers for high-fidelity amplification with *Pfu* DNA polymerase (Stratagene). *Pfu* PCR products were amplified from '*P. butanovora*' genomic DNA and sequenced directly to minimize errors due to DNA polymerase misincorporation. Discrepancies were resolved by sequencing multiple PCR products. Oligonucleotide primers were synthesized by Invitrogen. DNA sequencing was performed at the Center for Gene Research and Biotechnology Central Services Laboratory, Oregon State University. Sequence data were analysed using software from the GCG Wisconsin Package Version 10.0 (Acelerys). Sequence comparisons were made using the National Center for Biotechnology Information web site and BLAST (Basic Local Alignment Search Tool) (Altschul *et al*., 1997). Accession numbers of the sequences are listed in Table 2.

RNA was isolated from cells grown overnight to 0·8 OD_{600} with sodium lactate, a portion of which were washed,

Table 2. Sequence identity of butane monooxygenase with multicomponent monooxygenase proteins

Organism*	Hydroxylase, α subunit			Hydroxylase, β subunit			Hydroxylase, γ subunit			Regulatory protein			Reductase			Putative assembly		
	Protein	Size (aa)	ID (%)	Protein	Size (aa)	ID (%)	Protein	Size (aa)	ID (%)	Protein	Size (aa)	ID (%)	Protein	Size (aa)	ID (%)	Protein	Size (aa)	ID (%)
'*P. butanovora*'[a]	BMOX	530	100	BMOY	390	100	BMOZ	168	100	BMOB	137	100	BMOC	364	100	BMOD	104	100
M. capsulatus (Bath)[b]	MMOX	527	64	MMOY	389	42	MMOZ	170	38	MMOB	141	53	MMOC	348	39	MMOD	103	13
Methylocystis sp. M[c]	MMOX	526	64	MMOY	395	43	MMOZ	169	34	MMOB	138	54	MMOC	343	39	MMOD	111	13
M. trichosporium OB3b[d]	MMOX	525	63	MMOY	394	40	MMOZ	169	31	MMOB	138	53	MMOC	340	37	MMOD	102	14
R. rhodochrous B-276[e]	AMOC	501	32	AMOA	343	24	–	–	–	AMOB	117	18	AMOD	342	29	–	–	–
Xanthobacter Py2[f]	XAMOA	497	19	XAMOE	281	13	XAMOB	88	<10	XAMOD	101	<10	XAMOF	327	30	–	–	–
P. putida CF600[g]	DMPN	517	15	DMPL	331	16	DMPO	119	<10	DMPM	90	17	DMPP	353	28	DMPK	92	NS
Pseudomonas sp. JS150[h]	TBMD	513	13	TBMB	336	<10	TBME	121	<10	TBMC	89	<10	TBMF	355	26	TBMA	69	NS
P. mendocina KR1[i]	TMOA	500	15	TMOE	327	14	TMOB	84	<10	TMOD	103	15	TMOF	326	23	–	–	–
B. cepacia AA1[j]	TBHA	501	19	TBHE	332	<10	–	–	–	TBHD	104	18	TBHF	341	NS	–	–	–

NS, No significant similarity detected.

*Enzymes, accession numbers and references are as follows. *a*, Butane monooxygenase from '*Pseudomonas butanovora*', AY093933. *b*, Soluble methane monooxygenase from *Methylococcus capsulatus* (Bath), M90050 (Stainthorpe *et al*., 1990). *c*, Soluble methane monooxygenase from *Methylocystis* sp. M, U81594 (McDonald *et al*., 1997). *d*, Soluble methane monooxygenase from *Methylosinus trichosporium* OB3b, X55394 (Cardy *et al*., 1991). *e*, Alkene monooxygenase from *Rhodococcus rhodochrous* B-276, D37875 (Saeki & Keizo, 1994). *f*, Alkene monooxygenase from *Xanthobacter* Py2, AJ012090 (Zhou *et al*., 1999). g, Phenol hydroxylase from *Pseudomonas putida* CF600, M60276 (Nordlund *et al*., 1990). *h*, Toluene-2-monooxygenase from *Pseudomonas* sp. JS150, L40033 (Johnson & Olsen, 1995). *i*, Toluene-4-monooxygenase from *Pseudomonas mendocina* KR1, M65106 and M95045 (Yen & Karl, 1992; Yen *et al*., 1991). *j*, Toluene-3-monooxygenase from *Burkholderia cepacia* AA1, AF001356.

resuspended in basal medium, and subjected to an additional 3 h incubation with butane. Total RNA was isolated by the direct addition of acid-phenol, 100 mM sodium acetate and 1 % SDS to cell suspensions containing 4 mg cell protein. After thorough mixing, the suspension was centrifuged for 5 min at 16000 ***g***. The RNA was recovered by ethanol precipitation and dissolved in diethyl-pyrocarbonate-treated water. Approximately 5 μg total RNA was loaded in each lane of an agarose gel. Standard nucleic acid hybridization protocols were used (Sambrook *et al*., 1989). DNA probes were labelled by random priming using a kit (Prime-a-gene; Promega) and [α-^{32}P]dCTP (3000 Ci mmol^{-1}, $1{\cdot}1 \times 10^{14}$ Bq mmol^{-1}; ICN Biomedicals) following the directions of the manufacturers. DNA–DNA and RNA–DNA hybridization signals were visualized and analysed using phosphorimaging and ImageQuant software (Molecular Dynamics).

Generation of mutant strains. To generate a plasmid containing the complete sequence of *bmoX* for genetic manipulation, a *Pfu* (Stratagene) DNA polymerase PCR product was amplified from genomic DNA using the following primers: 5′-TGCTGATCTAGAACGAGATCGC-3′ and 5′-TCCAGGTCTAGATCAGATCAGAAAG-3′. To simplify cloning into pBluescript II SK(−), an *Xba*I restriction site was engineered into the PCR primers. The underlined nucleotides are altered from the actual *bmo* sequence. A 3410 bp *Xba*I restriction digest fragment of the product was cloned into the *Xba*I site of pBluescript II SK(−), resulting in the formation of plasmid pPbu281. To remove a *Bam*HI site in the vector, pPbu2 was constructed. For the inactivation of *bmoX*, the 4730 bp *lacZ*::kan^r cassette from pKOK6 (Kokotek & Lotz, 1989) was inserted into the *Bam*HI site of pPbu2 to obtain plasmid pPbu41 with the cassette in the orientation shown in Fig. 4. The site of insertion was at position 259 of the reported *bmo* sequence, which is located 60 nucleotides downstream of the ATG start codon of *bmoX* (see Fig. 4). The constructs were confirmed by DNA sequencing. The kanamycin-resistance constructs were introduced into '*P. butanovora*' by electroporation as described by Vangnai *et al*. (2002). Briefly, early stationary-phase '*P. butanovora*' cells (120 μl) in water were mixed with 1 μl plasmid DNA (0·5 μg) in a chilled electroporation cuvette (Invitrogen). Electroporation conditions were as follows: 1300 V, 71 μF and 200 Ω. After pulsing, the cells were first recovered in basal medium with citrate for 3 h at 30 °C and then challenged with 50 μg kanamycin sulfate ml^{-1}. After two days in liquid culture, the cells were selected on citrate-kanamycin plates.

RESULTS

Fractionation of butane monooxygenase into separate components

In vitro butane monooxygenase activity was reconstituted in the soluble portion of cell extracts by the addition of NADH. The inclusion of $Fe(NH_4)_2(SO_4)_2$, cysteine and glycerol in the lysis buffer increased the stability of the enzyme. Both ethylene and butane are substrates for sBMO (Hamamura *et al*., 1999). Ethylene oxide formation, a more sensitive assay than butane consumption or 1-butanol formation, was used to monitor enzymic activity. Fractionation of cell extracts by ion-exchange chromatography resulted in the lack of enzymic activity in any single fraction. We then investigated whether separate protein fractions could be recombined to restore butane-monooxygenase-catalysed ethylene oxidation. Substrate oxidation was observed only by combining three different protein components. No additional column fractions had an effect on the reconstituted activity in these assays.

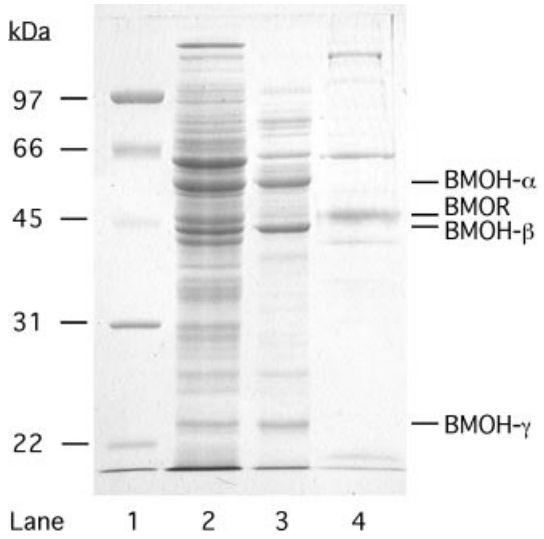

Fig. 1. SDS-PAGE analysis of partially purified butane monooxygenase hydroxylase (BMOH) and reductase (BMOR) components from '*P. butanovora*'. Lane 1, molecular mass standards (0·8 μg each); lane 2, cell extract (20 μg); lane 3, Q-Sepharose hydroxylase fractions (6·2 μg); lane 4, Q-Sepharose reductase fractions (3·9 μg).

Acetylene, a mechanism-based inactivator of butane monooxygenase, was used to target the component that contains the active site of substrate oxidation (Hamamura *et al*., 1999). Acetylene-inactivated extracts (see Methods) retained 1–5 % of the activity measured in untreated extracts. Only one of the protein components was able to restore ethylene oxidation activity to acetylene-inactivated extracts. Presumably, all necessary protein components except the active-site-containing component were not significantly affected by acetylene. The acetylene-sensitive component was enriched in three polypeptides with molecular masses of 54, 43 and 25 kDa, which are referred to as the α, β and γ subunits, respectively (Fig. 1). By analogy with the 'hydroxylase' components from multicomponent monooxygenases, this protein is referred to as the butane monooxygenase hydroxylase component (BMOH). The specific activity of BMOH for ethylene oxidation, as determined by restoration of activity to acetylene-inactivated extract, was 42 nmol (min mg hydroxylase protein)$^{-1}$. A second component exhibited a high rate of NADH:potassium ferricyanide oxidoreductase activity. This protein, referred to as the 'reductase' (BMOR), was enriched in a 45 kDa polypeptide. A third component (BMOB) was required for ethylene oxide activity in combination with the hydroxylase and the reductase, but did not exhibit any redox properties, nor was it able to restore activity to acetylene-inactivated extracts. This component was heat labile, but it was not noticeably enriched in any

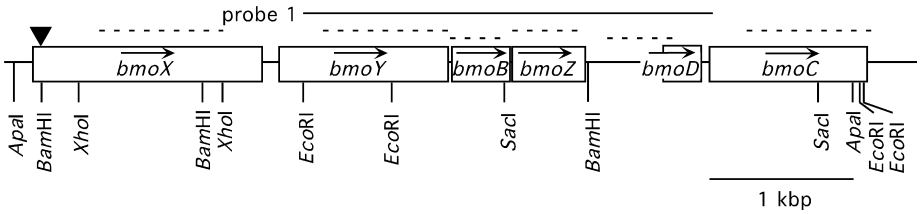

Fig. 2. Genetic loci of the butane monooxygenase genes in '*P. butanovora*'. The position of the insertional mutation in *bmoX* is indicated by an inverted triangle. Restriction sites are as predicted from the sequence and verified. The length and location of probes specific for each gene are indicated with dashed lines and probe 1 is indicated with a solid line.

particular peptides. Further purification and characterization is required to confirm our supposition that this component contains the small-molecular-mass 'regulatory protein' found in sMMOs (Fox *et al*., 1989; Green & Dalton, 1985), alkene monooxygenases (Miura & Dalton, 1995; Small & Ensign, 1997), and several aromatic monooxygenases (Newman & Wackett, 1995; Powlowski & Shingler, 1990).

Upon recombination, the three partially purified sBMO components possessed specific activity for ethylene oxidation of 27 nmol (min mg protein)$^{-1}$, a 2·9-fold purification above extract [9·3 nmol (min mg protein)$^{-1}$]. It is not surprising that this value is lower than the value obtained above with the hydroxylase component and acetylene-inactivated extracts given that the optimal ratio of the components in the assay has not been determined. The following N-terminal sequences were obtained from the partially purified proteins: STNIFTRGMVDPE (β subunit of BMOH), SKQVXYNTPV (γ subunit of BMOH), MLMQQYKIVARFEDG (BMOR).

Sequence analysis of the genes encoding sBMO

A 3010 bp PCR product was amplified from genomic DNA using degenerate oligonucleotide primers designed from the N-terminal sequences of the BMOH β subunit and the BMOR polypeptide (see Methods). The nucleotide sequence of this PCR product revealed a high degree of similarity to sMMOs (Cardy *et al*., 1991; Stainthorpe *et al*., 1990). The product was cloned and the insert was used to screen the '*P. butanovora*' genomic library (probe 1, Fig. 2). A 2993 bp portion of the *bmo* genes was sequenced from a genomic library clone, λPbu17 (Table 1), while the remaining upstream and downstream portions not present in the library clone were sequenced directly from high-fidelity PCR products amplified from genomic DNA. The sequence data of the assembled 6343 bp contig covers each strand with a minimum of threefold redundancy. The *bmo* structural genes (5797 bp) are ordered as shown in Fig. 2. The deduced amino acid sequences of the *bmoY*, *Z* and *C* genes (MSTNIFTRGMVDPE, MSKQVWYNTPV and MLMQQYKIVARFEDG, respectively) agreed with the N-terminal sequences obtained from the partially purified BMOH-β, BMOH-γ and BMOR peptides. Furthermore, the theoretical molecular masses (mentioned below) are comparable to the relative molecular masses determined by SDS-PAGE. Shine–Dalgarno-like ribosome-binding sites were found upstream of the *bmo* start sites by the following distances: 5 bases upstream of *bmoX* (GGAGG), 9 bases upstream of *bmoY* (GGAG), 9 bases upstream of *bmoZ* (GGAGG), 7 bases upstream of *bmoD* (GAGG) and 11 bases upstream of *bmoC* (GGAG). No clear ribosome-binding sequence was recognized upstream of *bmoB*.

BLAST searches against the sequence databases suggested that sBMO belongs to the family of enzymes with nonhaem carboxylate-bridged diiron sites (Wallar & Lipscomb, 1996). sBMO has significant sequence identity (Table 2) to methane monooxygenases, alkene monooxygenases, toluene monooxygenases and phenol hydroxylases, all of which are soluble monooxygenases composed of three or four components (Cardy *et al*., 1991; Miura & Dalton, 1995; Nordlund *et al*., 1990; Stainthorpe *et al*., 1990; Yen *et al*., 1991; Zhou *et al*., 1999). sBMO exhibited very little similarity to the integral-membrane liquid alkane hydroxylases of the diiron monooxygenase family, such as AlkB from *P. oleovorans* (Kok *et al*., 1989), AlkB from *Nocardioides* sp. CF8 (Hamamura *et al*., 2001), AlkM from *Acinetobacter* sp. ADP1 (Ratajczak *et al*., 1998) and XylM from *Pseudomonas putida* (Suzuki *et al*., 1991). No sequence similarity was detected between any of the sBMO proteins described in this work and the 54 kDa butane-induced polypeptide from *Pseudomonas* sp. IMT37 (Padda *et al*., 2001). Sequence alignments of butane monooxygenase with other monooxygenases revealed that the six sBMO gene products are all closest in amino acid sequence identity to peptides of the sMMOs (Table 2). The nomenclature for the sBMO genes and proteins shown in Table 2 is based primarily on the conservation of sequence and gene order with sMMO. The γ subunit of the sBMO hydroxylase does not have a counterpart in alkene monooxygenase from *Rhodococcus rhodochrous* B-276 or toluene-3-monooxygenase from *Burkholderia cepacia* AA1. Also, the *bmoD* gene product does not have an identifiable counterpart in alkene monooxygenases nor any of the aromatic monooxygenases. It aligned only with *mmoD* (previously *orfY*), which was recently found to be similar in properties to the *dmpK* gene product of phenol hydroxylase from *P. putida* CF600 (Merkx & Lippard, 2002). The implications of

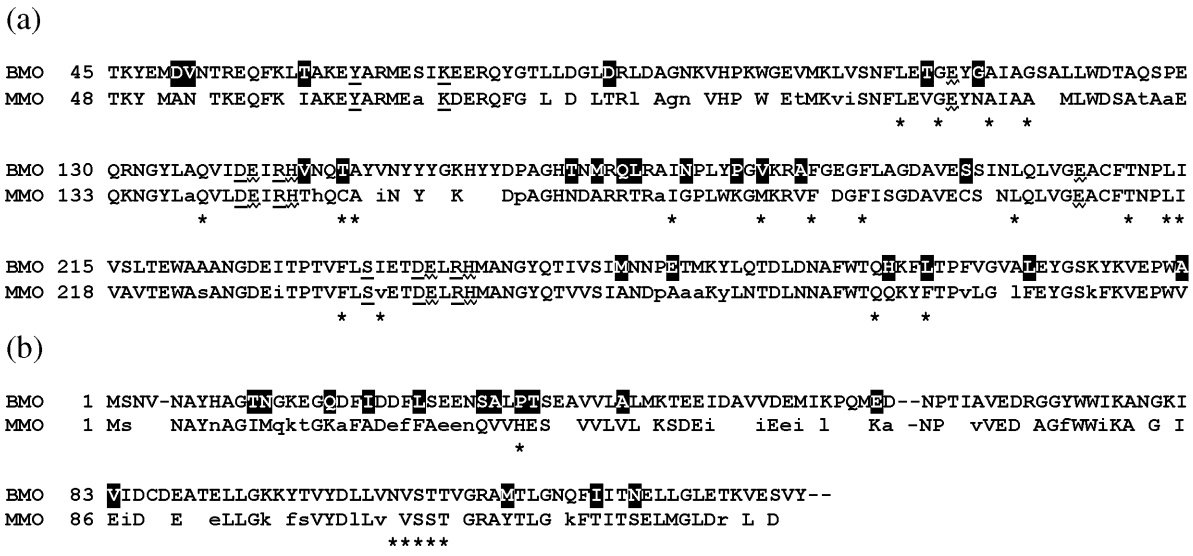

Fig. 3. Alignment of deduced amino acid sequences of sBMO with sMMO consensus sequences. (a) Comparison of hydroxylase α subunits; BMOH-α residues 1–44 (1–47, MMOH-α) and 300–530 (303–527, MMOH-α) are omitted for brevity. (b) Comparison of regulatory proteins. Residues that diverge from universally conserved sMMO residues are shaded black. The iron-binding ligands are underlined with wavy lines; the hydrogen bond network residues are underlined with straight lines. Asterisks below the sequences in (a) indicate the residues lining the hydrophobic pocket of MMOH from *Methylosinus trichosporium* OB3b and in (b) indicate H33 and the conserved residues at the core region of MMOB from *Methylosinus trichosporium* OB3b. Arrows point to divergent BMOH residues which align with the hydrophobic pocket residues. BMO: sBMO from '*P. butanovora*' (AY093933). MMO: consensus sequence of sMMO from *Methylococcus capsulatus* (Bath) (M90050; Stainthorpe *et al.*, 1990), *Methylosinus trichosporium* OB3b (X55394; Cardy *et al.*, 1991), *Methylocystis* sp. M (U81594; McDonald *et al.*, 1997), *Methylomonas* sp. KSWIII (AB025022; Shigematsu *et al.*, 1999), *Methylomonas* sp. KSPIII (AB025021; Shigematsu *et al.*, 1999), and *Methylocystis* sp. WI14 (AF153282; Grosse *et al.*, 1999). Upper case, identical in all six sMMO sequences; lower case, identical in at least four of the sequences; hyphen, gap in alignment; space, no consensus sequence.

the sequence similarity of sBMO to well-characterized systems are analysed in detail below.

The soluble BMOH component contains three subunits (α, β and γ) encoded by *bmoX*, *bmoY* and *bmoZ*, with theoretical molecular masses of 60·8, 45·0, 19·4 kDa, respectively. By analogy with other hydroxylase components, BMOH is likely to have an $\alpha_2\beta_2\gamma_2$ quaternary structure. The highest identity between sBMO and sMMO was found in the α subunits of BMOH and methane monooxygenase hydroxylase (MMOH) (63–64%). The α subunit contains the diiron active site of sMMO, where reduction of the metal centre, binding and subsequent cleavage of molecular oxygen, and substrate binding and hydroxylation all take place (Merkx *et al.*, 2001; Coufal *et al.*, 2000; Whittington & Lippard, 2001). By analogy with sMMO, the sBMO residues probably involved in coordinating the two irons are E111, E141, H144, E206, E240 and H243 (Fig. 3a). The residues involved in the H-bonding network of MMOH are conserved in BMOH (Coufal *et al.*, 2000; Whittington & Lippard, 2001). The universally conserved residues possibly involved in an analogous H-bonding network in sBMO are D140, R143, S235, D239, R242, Y64 and K71. Of the 457 amino acids that are identical or conserved among MMOH α-subunit sequences (Fig. 3a), 391 are conserved in BMOH-α. The remaining 66 BMOH amino acids that are not conserved at positions of high conservation among the six MMOHs are potentially of the most interest in terms of structure–function relationships. A 19-residue substrate-binding hydrophobic pocket adjacent to the diiron centre was identified from the crystal structure of MMOH from *Methylosinus trichosporium* (Elango *et al.*, 1997). Fourteen of the BMOH residues that align with the hydrophobic pocket residues are identical. Of the remaining residues, the three differences that seem most likely to influence the nature of the pocket are T148, V181 and L279, which align with C151, M184 and F282 of MMOH (Fig. 3a).

The deduced amino acid sequence of an ORF located between *bmoY* and *bmoZ* was found to be most similar to regulatory proteins that are required for enzymic activity in sMMOs (Table 2). This 15·1 kDa gene product is therefore referred to as the butane monooxygenase regulatory protein (BMOB). These proteins of sBMO and sMMOs shared the second highest sequence identity (53–54%) between the two systems. This protein has been studied extensively in sMMO and was found to regulate multiple steps occurring throughout the reaction cycle of sMMO (Wallar & Lipscomb, 2001). G13 of BMOB (G12 if the N-terminal methionine is processed) is identical to G13 of the methane

monooxygenase regulatory protein (MMOB) from *Methylococcus capsulatus* (Bath), which is partly responsible for the susceptibility of the sMMO regulatory protein to proteolysis and subsequent inactivation (Lloyd *et al*., 1997). BMOB has the extended N-terminus unique to sMMO that is lacking by 23 to 33 amino acids in all other multicomponent monooxygenase regulatory proteins. The N-terminus of MMOB was absolutely required for the ability of the protein to accelerate the MMOH reaction with O_2 (Chang *et al*., 2001). Of the 30 *Methylosinus trichosporium* OB3b MMOB residues which interact the most with reduced MMOH, 21 corresponding residues in BMOB are identical, 6 are similar, and only 3 are not conserved (H33 in MMOB is occupied by P32 in BMOB, A88 by C86, and T120 by N118). H33 of MMOB was found to be important in the formation of intermediate compound P, or a precursor, in the reaction with oxygen (Wallar & Lipscomb, 2001). The solution structure of MMOB from *Methylococcus capsulatus* (Bath) was solved and the residues which interact with the oxidized form of the hydroxylase were identified (Walters *et al*., 1999). The majority of the BMOB residues corresponding to these MMOB residues are for the most part, identical (F23, L93, G94, G111, R112 in BMOB) or similar (A26 of MMOB = S25 of BMOB, F100 = Y97, and D108 = N105). With respect to MMOB, two non-conservative substitutions in BMOB (F25 = L24, Y117 = M114) are at surface-exposed residues proposed to be involved in hydrophobic interactions with MMOH. The corresponding hydroxylase residues involved in these interactions have not been identified. Genetic analysis of MMOB from *Methylosinus trichosporium* OB3b also revealed that the core domain, consisting of residues 36–126, affects substrate access to, and product release from, the active site (Wallar & Lipscomb, 2001). BMOB contains a conservative substitution ($N^{105}VSTT^{109}$) with respect to MMOB from *Methylosinus trichosporium* OB3b ($N^{107}VSST^{111}$), retaining the bulky, polar character of this portion of the core domain (Fig. 3b). These residues were proposed to be involved in preferentially allowing access of small molecules for substrate-induced intermediate reactions at the active site of MMOH, a crucial aspect of the overall reaction also controlled by MMOB (Wallar & Lipscomb, 2001).

The reductase component (BMOR) has a theoretical molecular mass of 39·8 kDa and is encoded by *bmoC*. The sequence analysis and comparison (data not shown) reveals a [2Fe–2S] binding domain (ferredoxin domain) similar to those found in plants and bacteria (Lelong *et al*., 1995). The cysteine ligands to ferredoxin iron–sulfur centres are conserved in BMOR and located at residues 43, 48, 51 and 84 (42, 47, 50 and 82 in the methane monooxygenase reductase – MMOR – from *Methylococcus capsulatus*). A second domain (flavin domain) contains motifs for NADH-binding and flavin-binding sites (data not shown). The first solution structure of the ferredoxin domain of MMOR was recently solved from *Methylococcus capsulatus* (Bath) (Muller *et al*., 2002). The universally conserved G45 in MMOR that forms a hydrogen bond with S65 from the backbone glycine carbonyl is occupied by A46 in BMOR. Although the similarity between sBMO and sMMO is less for the reductases than the other components, several structural features of MMOR ferredoxin may be conserved in BMOR. For example, the sequences of a helix–proline–helix motif apparently unique to MMOR ferredoxins are identical or similar in BMOR: QVL (69–71) corresponds to QAL (67–69) of MMOR helix α_2, P73 of BMOR is also conserved, and DEEED (74–78) corresponds to EEEEE (72–76) involved in α_3 of MMOR. The MMOR α_2 helix was suggested to be essential for binding MMOH (Muller *et al*., 2002).

The 9·6 kDa *bmoD* gene product is similar in size and derived amino acid sequence to *mmoD* (*orfY*) (11·9 kDa). Merkx & Lippard (2002) have recently suggested that this conserved, yet previously uncharacterized, ORF in sMMO may be involved in assembly of the sMMO hydroxylase diiron centres. BMOD shares 13–14% identity with the MMOD proteins. Only 19% of MMOD residues are universally conserved. A gene found in the phenol hydroxylase cluster encodes a protein, DmpK, similar in size to MMOD. Studies with the overexpressed protein revealed that DmpK may help assemble the hydroxylase component of phenol hydroxylase by inserting iron into the apoprotein (Powlowski *et al*., 1997). DmpK-like proteins are found in other aromatic soluble diiron monooxygenases, including the *tbmA* gene product, which is 23% identical to DmpK (Johnson & Olsen, 1995). However, there is very little sequence similarity between these aromatic assembly proteins and BMOD or MMOD. In fact, only one residue is universally conserved among all of the proteins. If BMOD functions similarly to MMOD, then this conserved cysteine, C55, may also be involved in the interaction of the protein with the hydroxylase component during assembly of the metal centre (Merkx & Lippard, 2002).

Inactivation of the *bmoX* gene

We examined whether the *bmo* gene cluster was essential for growth on butane by mutagenesis of *bmoX*, which encodes the hydroxylase α subunit gene with the diiron active site. Insertional inactivation of *bmoX* was accomplished using a *lacZ*::kan^r cassette. The '*P. butanovora*' mutant (Pbu41) was unable to utilize butane as a growth substrate, whereas growth with lactate, citrate or 1-butanol was normal compared to wild-type growth. A low rate of ethylene oxidation [0·062 nmol (min mg protein)$^{-1}$] was exhibited by the mutant strain independent of prior exposure to butane. Incubations of the mutant strain for as long as 18 h with butane, to allow for induction of *bmo* gene expression, did not increase the rate of ethylene oxidation. This rate of ethylene oxidation in the mutant was not present in boiled cells and was approximately 0·4% of the ethylene oxidation rate present in butane-grown wild-type cells [15 nmol (min mg protein)$^{-1}$]. Butane did not induce the expression of the two 1-butanol dehydrogenases in the *bmoX*::*lacZ*::kan^r mutant, whereas incubation with 1-

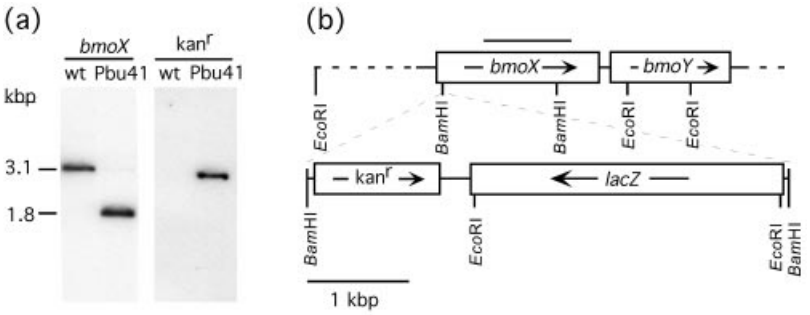

Fig. 4. (a) Southern blot analysis of '*P. butanovora*' DNA from wild-type and a *bmo* mutant strain. *Eco*RI-digested genomic DNA isolated from wild-type (wt) and mutant Pbu41 (Pbu41) was hybridized with a probe for *bmoX* or kan^r, as labelled. (b) Map of the 4730 bp *lacZ*::kan^r cassette (Kokotek & Lotz, 1989) showing the position in Pbu41. The location of the *bmoX* probe is indicated with a solid line.

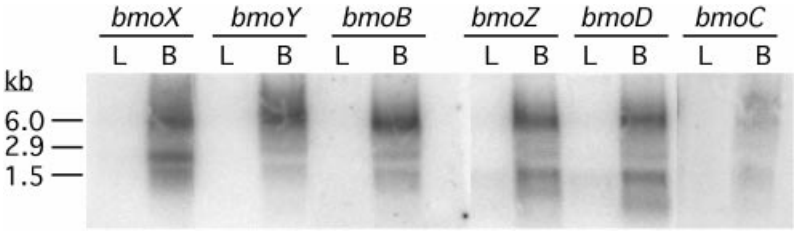

Fig. 5. Hybridization of '*P. butanovora*' RNA to probes for each of the *bmo* genes. RNA was extracted from cells grown with sodium lactate (lanes L) and from similar cells after an additional 3 h exposure to butane (lanes B). The migration of 23S and 16S rRNA in the gel is indicated by the 2·9 and 1·5 kb markers. The size of the large transcript detected with the probes (6·0 kb) was estimated from RNA size markers.

butanol induced 1-butanol dehydrogenase activity to wild-type levels in the mutant strain, as expected. The position of the cassette insertion in *bmoX* was confirmed by Southern blot analysis (Fig. 4).

Transcription of the *bmo* genes in response to butane

To examine the correlation between butane metabolism and expression of the *bmo* genes in '*P. butanovora*', RNA was isolated from washed lactate-grown cells before and after a 3 h exposure to butane. Probes for each of the six *bmo* genes hybridized to mRNA transcribed in response to butane (Fig. 5). The major transcript detected with all of the probes was approximately 6 kb in length. Shorter transcripts were also detected with the probes, but it is not known if these transcripts are functional products of processing or nonfunctional degradation products. The reason for the significantly lower levels of mRNA which hybridized to the probe for *bmoC* as compared to the intensity of the signal with the other five probes has not been determined. The length of the probe and specific activity of the label was comparable to the other five probes and the probe was effective in Southern blots. Samples of total RNA from cells grown with lactate and from cells induced with butane were loaded in approximately equivalent amounts, as judged by the intensity of nucleic acid staining in the agarose gel prior to blotting. In agreement with whole-cell studies of the induction of sBMO activity in '*P. butanovora*' (Sayavedra-Soto *et al.*, 2001), no *bmo* transcripts were detected in lactate-grown cells (Fig. 5). In addition, no transcripts were detected in the citrate-grown, butane-exposed mutant Pbu41 using probes for *bmoD*, *bmoC* or the short section of the *bmoX* gene upstream of the insertion site (data not shown).

Analysis of the DNA sequence upstream of the *bmo* genes revealed one region characteristic of promoter elements associated with σ^{54}-dependent transcription initiation (Barrios *et al.*, 1999). The putative promoter sequence is C^{102}ATGCTGGCACGACACTTGCTGA-A^{125}. The conserved nucleotides at the −24 and −12 positions are underlined. This sequence agrees with the consensus sequence of promoter elements recognized by the σ^{54}–RNA polymerase holoenzyme: mrNrYTGGCA-CGNNNNTTGCWNNw (Barrios *et al.*, 1999). In addition, the putative promoter element is identical to the consensus sequence identified for *Methylosinus trichosporium* OB3b, *Methylocystis* sp. M and *Methylomonas* sp. KSWIII (Murrell *et al.*, 2000). The nucleotide at the putative −12 element (C) is located 79 nucleotides upstream of *bmoX*.

DISCUSSION

The sBMO from '*P. butanovora*' was partially purified and six structural genes, *bmoXYBZDC*, were cloned and sequenced. The sequence analysis in conjunction with the requirements for activity in the partially purified system suggests that butane monooxygenase is a multicomponent enzyme composed of hydroxylase, regulatory protein, and a pyridine nucleotide (e.g. NADH) oxidoreductase. Acetylene is a mechanism-

based inactivator of sBMO, as well as sMMO, pMMO and ammonia monooxygenase (Hamamura *et al.*, 1999; Hyman & Wood, 1985; Prior & Dalton, 1985). The likely target of acetylene is the α subunit of the sBMO hydroxylase component, predicted to be 60 kDa from the sequence data. This subunit contains the absolutely conserved iron-binding motif of sMMO and related non-haem carboxylate-bridged diiron active sites (Nordlund *et al.*, 1992). A 58 kDa polypeptide was previously identified as a potential sBMO substrate-binding protein based on [^{14}C]acetylene labelling under turnover conditions in '*P. butanovora*' whole cells (Hamamura *et al.*, 1999; Sayavedra-Soto *et al.*, 2001). This result agrees with the suggestion that the *bmoX* gene encodes the substrate-binding subunit of the sBMO hydroxylase. The presence of a transcript which hybridized to the *bmoD* probe, together with the similarity of BMOD to MMOD, suggests the intriguing possibility that this protein may play a role in post-translational assembly of the hydroxylase metal centres.

The soluble multicomponent diiron monooxygenases require small-molecular-mass regulatory proteins which do not have any redox properties and do not have any organic or inorganic cofactors (Wallar & Lipscomb, 1996). Such a component does not participate in reactions catalysed by toluene dioxygenase (Subramanian *et al.*, 1985), xylene monooxygenase (Suzuki *et al.*, 1991) or alkane hydroxylase reactions (Kok *et al.*, 1989). The regulatory protein binds to the hydroxylase and controls the efficiency of the hydroxylase in sMMO systems in several ways, the most dramatic of which increases the rate of an intermediate reaction with O_2 by 1000-fold (Liu *et al.*, 1995). The high sequence identity (53 %) of BMOB with the MMOB from *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* (Bath) emphasizes the similarities between the two systems. The possibility that BMOB could substitute for MMOB in the interaction with the hydroxylase protein from sMMO is intriguing (Stirling & Dalton, 1979). The 'effector protein' of toluene-4-monooxygenase from *P. mendocina* (T4moD) could be replaced in the reconstituted system by the T3MO effector protein from *P. pickettii*, although with less affinity. But the effector proteins from *Pseudomonas* sp. JS150 or *B. cepacia* G4 T2MOs could not replace T4moD (Hemmi *et al.*, 2001). Those experiments examining the structures and functional groups of the regulatory proteins emphasized the high degree of specificity of the regulatory proteins for their respective hydroxylases.

We have not ruled out the possibility that an additional component may be involved in butane monooxygenase. Toluene-4-monooxygenase (Pikus *et al.*, 1996) and *Xanthobacter* Py2 alkene monooxygenase (Small & Ensign, 1997) are composed of four components, a result of separate ferredoxin and reductase redox components. The sequence of the sBMO oxidoreductase protein (BMOR) contains distinct binding site motifs for a [2Fe–2S] ferredoxin domain as well as pyridine-nucleotide- and flavin-binding motifs for a flavin domain. These features are typically found in oxidoreductases from three-component systems that do not require a separate ferredoxin. No ORF with similarity to ferredoxins was found either upstream or downstream of the *bmo* gene cluster. Together, these features of BMOR further distinguish BMO from membrane alkane hydroxylases, which utilize soluble rubredoxin and soluble NADH–rubredoxin reductase electron-transfer components. The likely role of the reductase in sBMO is to transfer electrons from NADH via FAD and the [2Fe–2S] ferredoxin to the diiron centre of the hydroxylase, analogous to the sMMO mechanism. Binding studies have revealed different types of interactions for the reductase and the regulatory protein with the hydroxylase component (Muller *et al.*, 2002; Walters *et al.*, 1999), providing further evidence of different binding sites on the sMMO hydroxylase for the two components (Gassner & Lippard, 1999).

'*P. butanovora*' is not capable of growth with sugars, methane or alkenes (Hamamura *et al.*, 1999; Takahashi *et al.*, 1980). Furthermore, '*P. butanovora*' does not appear to oxidize methane to methanol (Arp, 1999). Although sMMO catalyses the co-oxidation of butane, the products are predominantly 2-butanol, with smaller amounts of 1-butanol formed, whereas with '*P. butanovora*' sBMO, 1-butanol is the only detected product of butane oxidation (Arp, 1999). Given this difference, comparisons between methane monooxygenases and butane monooxygenase may provide insights into the source of substrate specificity of these homologous enzymes. sMMO is unique among the soluble monooxygenases in the ability to oxidize methane, and the reasons why are not clear. The explanation for the methane discrepancy may be found at the substrate-binding pocket, which for sMMO could provide the additional binding energy needed to oxidize methane, or help orient the substrate for reaction with oxygen, or, in conjunction with the regulatory protein, ensure that solvent is completely excluded from the active site. The surprisingly high homology of sBMO to sMMO may provide a framework for designing site-directed mutagenesis experiments to reveal the basis for this difference in catalytic activity between methane monooxygenases and the other soluble alkane monooxygenases.

The obligate requirement of the *bmo* gene cluster for butane metabolism was confirmed by mutagenesis of *bmo* genes. Given that 1-butanol induced wild-type levels of 1-butanol dehydrogenase activity in the sBMO mutant, it appears that the further metabolism of butane oxidation products is not disrupted. Growth with lactate, citrate or 1-butanol also was normal compared to wild-type growth. The residual level of ethylene oxidation detected in the mutant is similar to what was previously observed in catabolite-repression studies, where lactate- or lactate- and butane-grown wild-type cells possessed 0·3 % of the activity found in butane-grown wild-type cells (Sayavedra-Soto *et al.*, 2001). As suggested before, this activity may be due to a separate butane-sensing mechanism or to an additional alkane-oxidizing enzyme with limited specific activity for ethylene oxidation. This activity was not sufficient for

bacterial growth with butane, arguing against a catabolic role.

No other transcription initiation promoters were as clearly suggested from the sequence of the *bmo* genes as the putative σ^{54} promoter upstream of *bmoX*. Sequences with some similarity to both σ^{54} and σ^{70} promoter elements were present in the region upstream of *bmoY*, but no such similarities were identified in the *bmoD* or *bmoC* regions. With respect to the levels of MMOH present in extracts, MMOD and MMOR are expressed at molar ratios of 1·8 % in *Methylococcus capsulatus* and 10 % in *Methylosinus trichosporium*, respectively (Fox *et al*., 1989; Merkx & Lippard, 2002). This is consistent with correspondingly lower levels of mRNA for *mmoD* and *mmoC* present in extracts of wild-type *Methylococcus capsulatus* expressing sMMO (Nielsen *et al*., 1996). The *mmo* genes in *Methylococcus capsulatus* (Bath) seem to be co-transcribed from a single σ^{70} promoter element upstream of *mmoX* (Nielsen *et al*., 1996). However, in *Methylosinus trichosporium* OB3b, *Methylocystis* sp. M and *Methylomonas* sp. KSWIII putative σ^{54} promoters upstream of *mmoX* as well as an additional putative σ^{70} promoter in the *mmoY–mmoX* intergenic region of *Methylosinus trichosporium* may be involved in the transcription of *mmo* genes (McDonald *et al*., 1997; Murrell *et al*., 2000; Nielsen *et al*., 1997). Given that transcription start sites have been mapped to 11, 12 and 13 nucleotides, as well as other distances, downstream of the −12 promoter element (Barrios *et al*., 1999), the transcription start site of the *bmo* genes cannot be predicted from the promoter sequence. If the σ^{54}-like promoter element upstream of the *bmoX* gene is involved in recruiting σ^{54} to form σ^{54}–RNA polymerase holoenzyme, then the involvement of a σ^{54}-dependent enhancer binding protein in the initiation of transcription is expected. A comprehensive study of the transcription of the *bmo* genes involving mapping of the transcription start site, mutation of putative promoter sequences and transcriptional regulators will be required to understand the regulation of this multicomponent enzyme system.

The cloning and sequencing of the *bmo* genes will assist with mutagenesis for future studies of the complex regulation of the pathway of butane metabolism in '*P. butanovora*' as well as studies of the mechanism of butane oxidation. The purification and biochemical characterization of the three sBMO components is anticipated to augment the molecular characterization.

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