Molecular analysis of the soluble butane monooxygenase from \textit{Pseudomonas butanovora}’

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\textit{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). \textit{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 \textit{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 \textit{bmoXYZ}, a 40 kDa oxidoreductase encoded by \textit{bmoC}, and a 15 kDa regulatory protein encoded by \textit{bmoB}. A sixth structural gene (\textit{bmoD}) encodes a 9-6 kDa protein with similarity exclusively to \textit{mmoD (orfY)}, a putative metal centre assembly protein of the soluble methane monooxygenases. Insertional inactivation of \textit{bmoX} resulted in a mutant \textit{P. butanovora}’ strain incapable of growth with butane. A putative promoter element characteristic of promoters associated with \textit{\sigma}54-dependent transcription initiation was located upstream of the \textit{bmo} genes. Expression of all six genes was detected in butane-induced cells. Butane monooxygenase from \textit{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.

\textbf{Keywords:} butane metabolism, alkane oxidation, diiron monooxygenase

\section*{INTRODUCTION}

\textit{Pseudomonas butanovora}’ is capable of growth with alkanes (C$_2$–C$_n$), alcohols (C$_2$–C$_n$) and organic acids as sources of carbon and energy (Takahashi \textit{et al}., 1980). The 16S rRNA analysis suggests that the Gram-negative

\begin{itemize}
\item \textbf{Abbreviations:} BMOB, butane monooxygenase regulatory protein; BMOD, \textit{bmoD} gene product; BMOH, butane monooxygenase hydroxylase; BMOR, butane monooxygenase reductase; MMOD, methane monooxygenase regulatory protein; MMOD, \textit{mmoD (orfY)} gene product; MMOH, methane monooxygenase hydroxylase; MMOR, methane monooxygenase reductase; pMMO, particulate methane monooxygenase; sBMO, soluble butane monooxygenase; sMMO, soluble methane monooxygenase.
\end{itemize}

\textbf{The GenBank accession number for the \textit{bmoXYBZDC} sequence is AY093933.}

\textit{P. butanovora}’ is a \textit{\beta}-proteobacterium of the \textit{Rhodocyclus} group, related to members of the genus \textit{Thauera} (Anzai \textit{et al}., 2000). Butane metabolism in \textit{P. butanovora}’ proceeds by terminal hydroxylation of butane to 1-butanol. Subsequent oxidation reactions are first catalysed by butanol dehydrogenases to form butyraldehyde (Vangnai \textit{et al}., 2002), followed by oxidation of butyraldehyde to form butyrate (Arp, 1999), and probably metabolized further through the \textit{\beta}-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 \textit{et al}., 2002). Much less is known about the first enzyme in the catabolic pathway, butane monooxygenase. The butane monooxygenase of \textit{P. butanovora}’ has also been implicated...
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 monoxygenases 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 containing an integral membrane alkane hydroxylase, an NADH-oxidoreductase and a small regulatory protein (Colby & Dalton, 1978; Fox et al., 1979, 1981, 1985). Cells were typically harvested between 0–5 and 0.7 OD490. ‘P. butanovora’ was also cultured in a sealed 17 litre fermenter sparged with 7% butane (v/v) and periodic replenishments of O2 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(NH4)2(SO4)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 lystate was subjected to sonicaton with a microtip (Ultrasonics) for four cycles of 30 s pulses. The cell extract was clarified by ultracentrifugation (105 000 g for 1 h).

**Methods.** All chemicals used in this study were of analytical grade. Butane gas (99%) was purchased from Airgas. Acetyl- lene was generated by the addition of H2O to CaC2.

**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 CO2 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 harvested by centrifugation (10 min at 5000 g; 10 °C), washed by resuspension with 30 mM potassium phosphate (pH 7.2)–acetone 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(NH4)2(SO4)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 (Ultrasound) 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 monoxygenase (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 0.1–20 mg cell extract or column fractions. Acetylene, a mechanism-based inactivator of butane monoxygenase (Hamamura et al., 1999), was used to inactivate the substrate–binding component of butane monoxygenase. 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 monoxygenase (sBMO) components in the activity assays where indicated.

**Separation of the butane monoxygenase 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 monoxygenase activity were pooled, concentrated, and desalted by affinity chromatography using CM-Sepharose. The concentration of the A component was determined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970) using a 10% gel followed by staining with Coomassie Brilliant Blue R-250. Gel slices were vacuum-dried and homogenized in 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% SDS and 1% β-mercaptoethanol. These protein digests were separated by SDS-PAGE, and the protein bands were visualized by silver staining (Huff et al., 2000).
monooxygenase activity by complementing the hydroxylase and reductase components were pooled. The second component, proposed to be the hydroxylase component, eluted between 320 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 Rf values of standard proteins. The partially purified components were concentrated by ultrafiltration (Millipore) and reductase components were pooled. The second component, proposed to be the hydroxylase component, eluted between 400 and 360 mM NaCl and the third component, eluted between 320 and 360 mM NaCl.

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 Rf values of standard proteins. The partially purified components were concentrated by ultrafiltration (Millipore; YM100 for the hydroxylase component, YM10 for the reductase component).

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’-ATCTTCCAACCGCGGATGGTN-GAYCCNGA-3’ and the reverse primer sequence was 5’-GGCCAGATCTTTGTACTGTYGCATNARC-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 EcoRI or BamHI, 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 OD600 with sodium lactate, a portion of which was washed,

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant properties</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Pseudomonas butanovora’</td>
<td>Wild-type</td>
<td>ATCC</td>
</tr>
<tr>
<td>ATCC 43655</td>
<td>ATCC 43655 derivative, bmoX::lacZ::kan’ cassette inserted at BamHI site (position 259)</td>
<td>This study</td>
</tr>
<tr>
<td>Pbu41</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>endA1 recA1 gyrA96 thi bsdR17 (r6 m3) relA1 supE44 (Δlac–proAB) [F’ traD36 proAB lacPZΔM15]</td>
<td>Promega (Yanisch-Perron et al., 1985)</td>
</tr>
<tr>
<td>JM109</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>LE392</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>Plasmids and λ clones</td>
<td>bmoX::lacZ::kan’ cassette inserted at BamHI site (position 259)</td>
<td>This study</td>
</tr>
<tr>
<td>λPbu17</td>
<td>PCR product (3-4 kbp) with introduced flanking XbaI sites containing complete bmoX and partial bmoY; cloned in the XbaI site of pBluescript II SK (–)</td>
<td>This study</td>
</tr>
<tr>
<td>pPbu2</td>
<td>pPbu281 subclone</td>
<td>This study</td>
</tr>
<tr>
<td>pPbu41</td>
<td>pPbu2 subclone with a lacZ::kan’ cassette inserted at bmoX BamHI site (position 259)</td>
<td>This study</td>
</tr>
<tr>
<td>Cloning vectors</td>
<td>λ vector used to construct the genomic library of ‘P. butanovora’</td>
<td>Promega</td>
</tr>
<tr>
<td>λ-Bluescript II SK (–)</td>
<td>2961 bp phagemid derived from pUC19; multiple cloning cassette; Amp’</td>
<td>Stratagene; GenBank no. X52330</td>
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<tr>
<td>pBluescript II SK (–)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGEM-T Easy</td>
<td>PCR product cloning vector; multiple cloning cassette includes flanking EcoRI sites; Amp’</td>
<td>Promega</td>
</tr>
</tbody>
</table>

Table 1. Bacterial strains and plasmids used in this work

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

<table>
<thead>
<tr>
<th>Organism</th>
<th>Hydroxylase, α subunit</th>
<th>Hydroxylase, β subunit</th>
<th>Hydroxylase, γ subunit</th>
<th>Regulatory protein</th>
<th>Reductase</th>
<th>Putative assembly</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein Size (aa)</td>
<td>ID (%)</td>
<td>Protein Size (aa)</td>
<td>ID (%)</td>
<td>Protein Size (aa)</td>
<td>ID (%)</td>
</tr>
<tr>
<td>'P. butanovora'</td>
<td>BMOX 530 100 BMOY 390 100 BMOZ 168 100</td>
<td>BMOB 137 100 BMOC 364 100 BMOD 104 100</td>
<td>M. capsulatus (Bath)</td>
<td>MNOX 527 64 MNOY 389 42 MNOZ 169 34</td>
<td>MMOB 141 53 MMOC 348 39 MMOD 103 13</td>
<td>MMOX 527 64 MMOY 389 42 MMOZ 169 34</td>
</tr>
<tr>
<td>Methylocystis sp.</td>
<td>MNOX 526 64 MNOY 395 43 MNOZ 169 34</td>
<td>MNOB 138 54 MMOC 343 39 MMOD 111 13</td>
<td>M. trichosporium OB3b</td>
<td>MNOX 525 63 MNOY 394 40 MNOZ 169 31</td>
<td>MMOB 138 53 MMOC 340 37 MMOD 102 14</td>
<td>MMOX 525 63 MMOY 394 40 MMOZ 169 31</td>
</tr>
<tr>
<td>R. rhodochrous B-276</td>
<td>AMOC 501 32 AMOA 343 24</td>
<td>AMOB 117 18 AMOD 342 29</td>
<td>Xanthobacter Py2</td>
<td>XAMOA 497 19 XAMOE 281 13 XAMOB 88 &lt;10 XAMOD 101 &lt;10 XAMOF 327 30</td>
<td>– – – – – – – – – – – – – –</td>
<td>– – – – – – – –</td>
</tr>
<tr>
<td>P. putida CF600</td>
<td>DMPN 517 15 DMPL 331 16 DMPO 119 &lt;10 DMPM 90 17 DMPP 353 28 DMPP 92</td>
<td>ns</td>
<td>Pseudomonas sp. JS150</td>
<td>TBMD 513 13 TBMB 336 &lt;10 TBME 121 &lt;10 TBM 89 &lt;10</td>
<td>69</td>
<td>ns</td>
</tr>
<tr>
<td>P. mendocina KR1</td>
<td>TMOA 500 15 TMOE 327 14 TMOB 84 &lt;10 TMO 103 15 TMO 326 23</td>
<td>– – – – – – – –</td>
<td>B. cepacia AA1</td>
<td>TBHA 501 19 TBHE 332 &lt;10 – –</td>
<td>– – – – – –</td>
<td>– – – – – –</td>
</tr>
</tbody>
</table>

ns, No significant similarity detected.

resuspended in basal medium, and subjected to an additional 3 h incubation with butane. Total RNA was isolated by the direct extraction 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-32PdCTP (3000 Ci mmol−1, 1.1 × 108 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).

**RESULTS**

**Fractionation of butane monoxygenase into separate components**

In vitro butane monoxygenase activity was reconstituted in the soluble portion of cell extracts by the addition of NADH. The inclusion of Fe(NH4)2(SO4)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- monoxygenase-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.

Acetylene, a mechanism-based inactivator of butane monoxygenase, 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 ‘reductase’ (BMOR), was enriched in a ferricyanide oxidoreductase activity. This protein, referred to as the ‘hydroxylase’ (BMOH), was enriched in 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...
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; Powlsowski & 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: STNIFTR-GMVDPDE (β subunit of BMOH), SKQVXYNTTPV (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, xPbu17 (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 (MSTNIFTRGMVDPDE, MSKQVWXNTVP 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 non-haem 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; Yun 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 Nocardoides 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
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 α#β#γ# 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 OB3b (Elango et al., 1997). Fourteen of the BMOH residues that 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 (K5394; Cardy et al., 1991), Methylocystis sp. M (UB1594; McDonald et al., 1997), Methylophilus sp. KSWII (AB025021; Shigematsu et al., 1999), Methylophilus sp. KSII (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.
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). MMOB 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$^{105}$VSST$^{109}$) with respect to MMOB from *Methylosinus trichosporium* OB3b (N$^{105}$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 *Methyloccoccus 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 x$_2$, P73 of BMOR is also conserved, and DEEED (74–78) corresponds to EEEE (72–76) involved in x$_2$ of MMOR. The MMOR x$_2$ 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 (Pawlowski 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 χ subunit with the diiron active site. Insertional inactivation of *bmoX* was accomplished using a *lacZ::kan*' 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*' mutant, whereas incubation with 1-
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 \( \text{kbp} \) 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 \( \sigma^{34} \)-dependent transcription initiation (Barrios et al., 1999). The putative promoter sequence is C\(^{102}\)ATGCTGCGACGACACTTGCTAGA\(^{125}\). The conserved nucleotides at the \(-24\) and \(-12\) positions are underlined. This sequence agrees with the consensus sequence of promoter elements recognized by the \( \sigma^{34} \)-RNA polymerase holoenzyme: mrNrYTGCGACGNNNTTG\(^{w}\) (Barrios et al., 1999). In addition, the putative promoter element is identical to the consensus sequence identified for \( Methyllosinus \) 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 ζ 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 38 kDa polypeptide was previously identified as a potential sBMO substrate-binding protein based on [13C]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 O₂ by 1000-fold (Liu et al., 1995). The high sequence identity (53%) of BMOD with the MMOD from Methylosinus trichosporium OB3b and Methylococcus capsulatus (Bath) emphasizes the similarities between the two systems. The possibility that BMOD could substitute for MMOD 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 alkane monooxygenase (Small & Ensign, 1997) are composed of four components, a result of separate ferredoxin and reductase redox components. The sequence of the sBMO oxidoeductase 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 oxidoeductases 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- and 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 catalytic 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 trichosporum, 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 trichosporum 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 trichosporum 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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