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Product and product-independent induction of butane oxidation in *Pseudomonas butanovora*

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Abstract

Pseudomonas butanovora grows on butane by means of an inducible soluble alkane monooxygenase (sBMO). The induction of sBMO was studied using the wild type and a sBMO reporter strain. The reporter strain has the *lacZ::kan* cassette inserted into *bmoX*, the gene that encodes the α-subunit of the hydroxylase of sBMO. The β-galactosidase activity in the reporter strain was not induced by butane, but was induced by 1-butanol and butyraldehyde. *P. butanovora* expressed sBMO product-independent activity at 3.0 ± 1 nmol ethylene oxide min^{-1} mg $protein^{-1}$ in stationary phase. The sBMO product-independent activity likely primes the expression of sBMO by butane.
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Keywords: *Pseudomonas butanovora*; Soluble butane monooxygenase; Induction of sBMO; sBMO reporter strain

1. Introduction

Pseudomonas butanovora (ATCC 43655) is an aerobic Gram-negative bacterium that is able to grow on *n*-alkanes (C2–C9) as sole sources of carbon and energy [1]. The 16S rDNA nucleotide sequence of *P. butanovora* suggested that this bacterium is phylogenetically affiliated to the genera *Thauera* and *Azoarcus* [2] rather than to the genus *Pseudomonas*.

Pseudomonas butanovora utilizes butane (C4 *n*-alkane) by inducing the expression of a soluble alkane monooxygenase, sBMO. The enzyme catalyzes the oxidation of butane to 1-butanol. The 1-butanol is oxidized to butyraldehyde by two inducible alcohol dehydrogenases [3,4]. Butyraldehyde is oxidized to butyric acid by at least two aldehyde dehydrogenases, and is then further metabolized through the β-oxidation pathway of fatty acid metabolism [5].

The first three enzymes in the pathway have been characterized genetically and biochemically [3,4,6]. The first enzyme, sBMO, is encoded by six structural genes and is a multicomponent enzyme composed of a hydroxylase, a regulatory protein, and a pyrimidine nucleotide oxidoreductase. The protein structure and gene arrangement of sBMO in *P. butanovora* is very similar to the soluble monooxygenase (sMMO) from methanotrophs [6]. Two alcohol dehydrogenases are involved in the second step and are PQQ-containing enzymes (a quinoprotein

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and a quinohemoprotein). The two alcohol dehydrogenases are directly linked to butane metabolism. The simultaneous inactivation of the genes for the alcohol dehydrogenases impeded the growth of *P. butanovora* on butane [3,4]. The involvement of dedicated alcohol dehydrogenases in alkane metabolism has been reported for other microorganisms [7,8].

Methane, gaseous alkanes (C2–C5) and liquid alkanes (C6) can serve as growth substrates for bacteria [9–13]. The monooxygenase from *P. butanovora* shares more commonalities structurally with the methane oxidizers [14–16] than with the liquid alkane oxidizers. In contrast, the regulation of sBMO expression shares more commonalities with the liquid alkanes oxidizers. The enzymes that catalyze the oxidation of liquid alkanes are different in composition from the monooxygenases for gaseous alkane utilizers. For example, in *Pseudomonas putida* GPO1, a membrane-associated binuclear iron-containing alkane hydroxylase is produced in response to C6–C12 alkanes [17–23]. In *Acinetobacter* ADP1, an integral-membrane hydroxylase initiates the metabolism of $\geqslant$C12 alkanes [24,25]. The hydroxylase of *P. putida* GPO1 and *Acinetobacter* ADP1 are regulated by interaction of regulatory elements with the alkane substrates [26,27]. Two distinct monooxygenases are present in most methane oxidizers. These monooxygenases are differentially regulated by Cu. Under conditions of Cu limitation, a soluble methane monooxygenase is produced [14] and under conditions of Cu sufficiency, a particulate monooxygenase is produced [16].

The induction profiles of the enzyme involved in the utilization of butane in *P. butanovora* were previously documented [28]. The enzymes were induced in response to butane and its oxidation metabolites. Cells grown on metabolites of butane oxidation readily consumed the metabolites as well as the downstream products. However, these cells consumed the precursors of the growth metabolite more slowly. In this communication, we use the wild-type *P. butanovora* and the sBMO reporter strain *P. butanovora bmoX::lacZ::kan* to further understand the induction of sBMO activity.

2. Materials and methods

2.1. Strains and growth conditions

Pseudomonas butanovora (ATCC 43655) was cultured in sealed bottles (720 ml) containing 100 ml of liquid medium and 620 ml of air, with 30 ml of butane gas (99.0%; Airgas, Inc., Randor, PA) added as an overpressure. The alternative energy and carbon sources (sodium citrate or sodium lactate) were added at 5–10 mM concentration. The basic growth medium was optimized from [29] and consisted of 2 mM $MgSO_4 \cdot 7H_2O$, 400 μM $CaCl_2 \cdot 2H_2O$, 0.05 mM $FeSO_4$, 60 mM NH_4Cl, phosphate buffer (7 mM $Na_2HPO_4 \cdot 7H_2O$, and 15 mM KH_2PO_4; pH 7.2), 0.01 g/l yeast extract and the trace elements described [30]. The sBMO reporter *P. butanovora* mutant strain *bmoX::lacZ::kan* has the *lacZ::kan* cassette disrupting *bmoX*. *P. butanovora bmoX::lacZ::kan* is BMO-deficient and resistant to kanamycin and was produced by electroporation of the donor plasmid pB21 into *P. butanovora* wild type as described [3,6]. Plasmid pB21 has the *lacZ::kan* cassette from pKOK6 [31] inserted into *bmoX*, the gene that encodes the α subunit of the hydroxylase component of sBMO. The cells were recovered in basal medium with sodium citrate for 3 h at 30 °C and then challenged with 20 μg of kanamycin sulfate ml^{-1}. A loop of the culture was streaked onto a kanamycin plate and a single colony was used to propagate a clonal culture for the experiments. The correct gene inactivation was corroborated by Southern hybridization.

2.2. Nucleic acid manipulations

DNA manipulations were performed using standard protocols [32]. DNA-free RNA was prepared using a Bio-Rad (Hercules CA, USA) kit. Quantitative RT-PCR (qRT-PCR) was performed on an iCycler iQ single color Real-Time PCR detection system with an iQ SYBR Green Supermix (Bio-Rad). Data were analyzed with Gene Expression Macro version 1.1 (Bio-Rad). Primers were designed to amplify 200–300 bp of the open reading frames coding for sBMO.

2.3. Activity assays

Cells of *P. butanovora* were harvested at an OD of approximately 0.5, washed twice in basal medium and suspended to a similar OD as at the time of harvest. For most experiments, the cell suspensions were incubated 3 h in either 10% (headspace) butane, 2 mM 1-butanol, 2 mM butyraldehyde or 2 mM sodium butyrate as indicated.

The activity of sBMO in whole cells was measured in 10 ml sealed serum vials by the oxidation of ethylene, an alternative substrate for sBMO, to ethylene oxide [6,28]. Protein concentration was determined by the Biuret assay with serum albumin as standard [33].

The β-galactosidase activity in *P. butanovora bmoX::lacZ::kan* was measured using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) [34]. *P. butanovora bmoX::lacZ::kan* cells were harvested, washed once in basal medium, and 1 ml of cell suspension (~0.01 OD) was assayed. The inducers were added at the indicated concentrations and incubated for 2 h. At the end of the induction treatment, the development of color was initiated by the addition of ONPG and stopped by the addition of $NaHCO_3$ (1%, v/v). The β-galactosidase activity was

calculated as described [35]. Citrate in the medium was measured using a commercial kit from Boehringer–Mannheim/Roche (Germany).

3. Results

3.1. Reporter strain characterization

The reporter strain of *P. butanovora* was constructed to analyze the activation of the *bmo* promoter (P*bmo*). Two kanamycin-resistant mutants of *P. butanovora*, *bmoX::lacZ::kan* (forward orientation) and *bmoX::kan::lacZ* (reverse orientation), were isolated. The correct insertion point and orientations were corroborated by Southern hybridization and phosphorimaging (Fig. 1). The insertion of the cassette into *bmoX* rendered *P. butanovora* unable to utilize butane for growth but still capable of growing on citrate, lactate, 1-butanol and butyraldehyde. The wild-type strain and both mutant strains were tested for β-galactosidase activity. Only *P. butanovora bmoX::lacZ::kan* induced β-galactosidase activity when subjected to the compounds described below. The *P. butanovora bmoX::lacZ::kan* strain was used to characterize the expression of sBMO.

3.2. Induction of Pbmo

Wild-type cells, when incubated in butane or in butane and citrate, induce sBMO activity within 2 h (Fig. 2 and [5,28]). However, 1-butanol and butyraldehyde were more efficient in inducing sBMO activity, whereas sodium butyrate was not effective. To test if butane was indeed an inducer of sBMO, we incubated *P. butanovora bmoX::lacZ::kan* cells in the presence of butane, 1-butanol, butyraldehyde, or sodium butyrate. Surprisingly, butane, the primary substrate of sBMO, did not induce P*bmo* to express β-galactosidase activity (Fig. 2). On the other hand, incubations with either

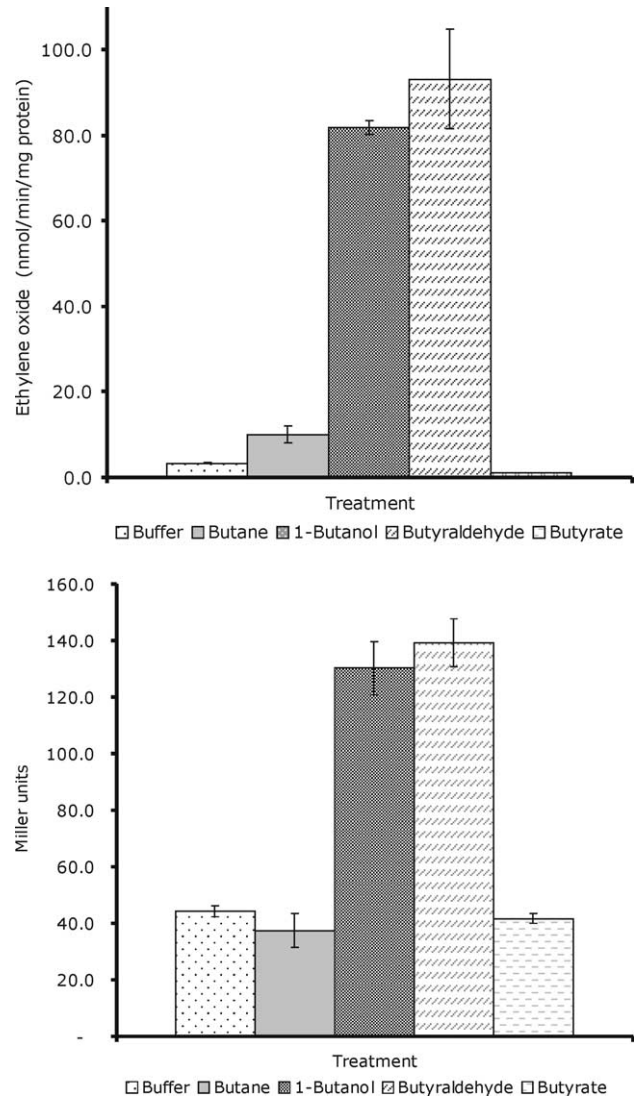

Fig. 2. Induction of ethylene oxidation in *P. butanovora* cells (top) and β-galactosidase activity in *P. butanovora bmoX::lacZ::kan* (bottom) by the substrates indicated. Cells were grown on sodium lactate, harvested in the stationary phase and washed before the 2.5 h incubation in the substrate indicated.

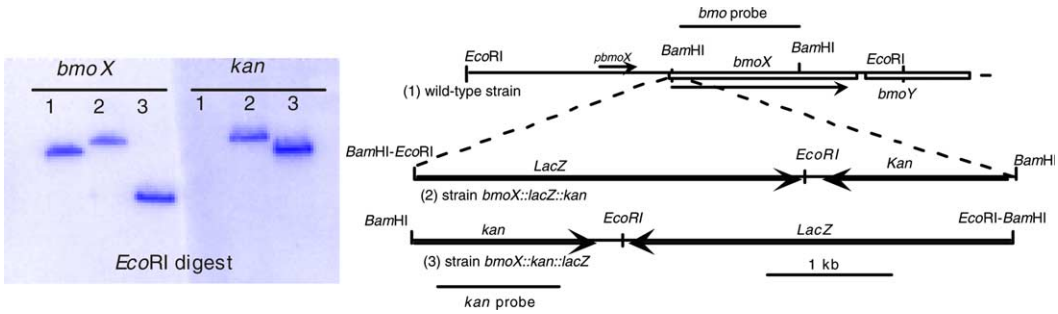

Fig. 1. Southern hybridization (left) and map (right) of the insertion of a *lacZ::kan* cassette into *bmoX*. The DNA from two mutant strains in *bmoX* was used and shows the two possible orientations of the cassette. The hybridizations are to the *bmoX* probe and *kan* probe. Lane 1: wild-type *P. butanovora*; lane 2: strain *bmoX::lacZ::kan* (forward orientation); lane 3: strain *bmoX::kan::lacZ* (opposite orientation). The same blot was used for both hybridizations.

1-butanol or butyraldehyde induced β-galactosidase activity in *P. butanovora bmoX::lacZ::kan*. In most instances butyraldehyde induced up to 10% more sBMO activity or β-galactosidase activity than 1-butanol. These results suggest that P*bmo* is activated by two of the oxidation products of butane.

If 1-butanol and butyraldehyde are the actual inducers of sBMO activity, it is necessary for the bacterium to possess enough sBMO activity to oxidize butane to 1-butanol to start the induction of P*bmo*. Indeed, there is precedent in *P. butanovora* for a relatively low sBMO activity when grown on substrates other than butane or its immediate oxidation products [28]. In wild-type cells, this activity was detected by following the accumulation of ethylene oxide in sealed vials or in [$U^{14}C$]-acetylene sBMO labeling experiments [28], using a non-optimized growth medium. Butane-grown cells typically develop activities between 20 and 100 nmol EtO mg protein^{-1} min^{-1} depending on the growth conditions [6,36].

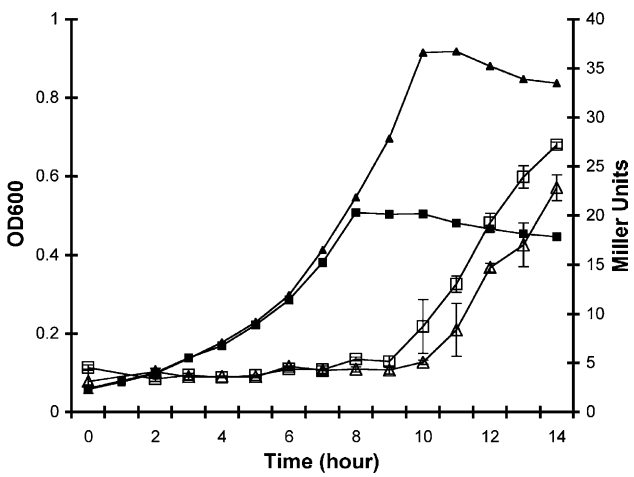

Fig. 3. Growth phase product-independent activity on sodium citrate grown *P. butanovora bmoX::lacZ::kan* as measured by β-galactosidase activity. Growth of culture with 5 mM sodium citrate (black square symbols) with corresponding β-galactosidase activity (white square symbols); growth with 10 mM sodium citrate culture (black triangle symbols) with corresponding β-galactosidase activity (white triangle symbols).

3.3. Product-independent induction of Pbmo

Of significance to the present observation that products induced sBMO activity is the fact that wild-type cells grown with citrate or lactate showed sBMO activity (3.0 ± 1 nmol EtO mg protein^{-1} min^{-1}). In *P. butanovora*, the activity that develops without the addition of butane or any of its metabolites we refer to as product-independent sBMO activity. This product-independent activity was seen consistently in the reporter strain *bmoX::lacZ::kan* (20–40 Miller units; e.g. Fig. 2, buffer treatment and Fig. 3). In the wild-type strain and reporter strain *bmoX::lacZ::kan*, product-independent activity was detected at higher levels when the cells entered the stationary phase. We proceeded to characterize the development of this activity during growth using the reporter strain *bmoX::lacZ::kan* (Fig. 3). In all instances, the induction of the product-independent activity developed to higher levels in the stationary phase, triggered by the depletion of the carbon source (Table 1). The product-independent activity developed to higher levels when either sodium lactate (Fig. 2) or sodium citrate (Fig. 3 and Table 1) was depleted from the growth medium. Cells grown in nitrogen limited medium, but with sufficient carbon, did not develop higher product-independent activity in the stationary phase (Table 1).

We determined the response of wild-type cells to butane in relatively short incubations by qRT-PCR. The mRNA for *bmoX* accumulated in a 25 min incubation. This suggests that the product-independent activity is sufficient to prime the expression of sBMO. However,

Table 2
Fold induction[a] sBMO mRNA in sodium citrate grown *P. butanovora* upon exposure to butane

Time (min)	Cells in logarithmic growth	Cells in stationary phase
0	1.00 ± 0.11	5.19 ± 1.84
10	1.52 ± 1.14	5.74 ± 0.33
25	2.33 ± 0.27	23.24 ± 2.35

[a] Fold increase with respect to the levels at time zero in cells during logarithmic growth.

Table 1
Product-independent activity in *P. butanovora bmoX::lacZ::kan* cultures limited for carbon (sodium citrate) or nitrogen (NH_4Cl) at stationary phase

Nitrogen	Carbon	5 mM	7 mM	10 mM
30.0 mM	Activity	27.3 ± 6.4 units[a]	33.6 ± 5.3 units	36.7 ± 1.3 units
	Final OD	0.40	0.59	0.75
	Citrate left	⩽0.01 mM[b]	⩽0.01 mM	⩽0.01 mM
0.05 mM	Activity	2.3 ± 0.2 units	2.4 ± 1.7 units	2.6 ± 1.4 units
	Final OD	0.31	0.30	0.30
	Citrate left	0.86 ± 0.06 mM	3.56 ± 0.09 mM	4.43 ± 0.42 mM

[a] Miller units.
[b] Lowest detection limit for the assay.

the *bmoX* mRNA levels accumulated to higher levels in stationary phase cells than in logarithmic phase cells (Table 2).

4. Discussion

Here we show that the inducer for sBMO is not butane, its natural substrate, but two of the products of the oxidation of butane, 1-butanol and butyraldehyde. For the oxidation of butane, *P. butanovora* has the following attributes. One, the activities of the two 1-butanol alcohol dehydrogenases are not present in the absence of their substrates [3]. Two, a relatively low constitutive aldehyde dehydrogenase activity is present in the absence of the substrate [28]. Three, the enzymes carrying out the oxidation of 1-butanol and butyraldehyde are induced to higher levels by their corresponding natural substrate [28]. Four, *P. butanovora* uses the product-independent sBMO activity to produce 1-butanol and butyraldehyde to prime the activation of P*bmo*. Five, the butyraldehyde produced by the induced alcohol dehydrogenases would be rapidly consumed but still lead to activation of P*bmo*. This strategy permits *P. butanovora* to accumulate 1-butanol to activate P*bmo*, while still not accumulating any butyraldehyde that the alcohol dehydrogenases might produce. Butyraldehyde is toxic at high levels to the cell [4]. The end result is the full induction of sBMO activity (Figs. 2 and 4). Evidence of this is that *P. butanovora* responds to butane in the first 10 min upon exposure by producing de novo BMO mRNA (Table 2). The product-independent sBMO activity is sufficient to produce the necessary 1-butanol to prime the induction of P*bmo*.

When the source of carbon becomes limiting, *P. butanovora* uses resources to ready itself for the next available carbon substrate. It was in this state that the product-independent sBMO activity increased. The depletion of carbon source (Fig. 3 and Table 1) prompts the cell to develop higher product-independent activity. This phenomenon has been observed in other organisms [37,38]. When carbon is scarce, the cells prepare for a quick response to a new carbon source when it becomes available. Indeed, this level of product-independent sBMO activity made *P. butanovora* produce more mRNA when butane was supplied to stationary phase cells than to logarithmic phase cells (Table 2). In *P. butanovora*, butane oxidation is initiated through the product-independent sBMO activity to produce the 1-butanol necessary to enhance the activation of P*bmo* (Figs. 3 and 4). In energy limiting conditions, the low product-independent sBMO activity may help the bacterium to react to the alkane without wasting valuable energy resources.

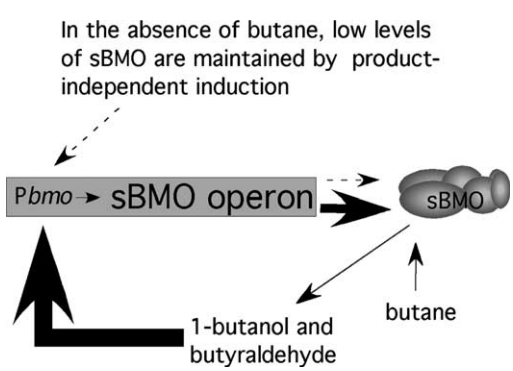

Fig. 4. Model for the activation of P*bmo*. In the absence of butane (dashed arrows), low levels of sBMO are maintained. When butane is present, the expression levels are enhanced by the 1-butanol and butyraldehyde produced (thick arrows).

Acknowledgments

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