

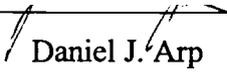
AN ABSTRACT OF THE THESIS OF

Alisa S. Vangnai for the degree of Doctor of Philosophy in Biochemistry and Biophysics presented on May 17, 2002.

Title: Biochemical, Molecular and Physiological Characterization of 1-Butanol Dehydrogenases of *Pseudomonas butanovora* in Butane and 1-Butanol Metabolism.

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Abstract approved: _____


Daniel J. Arp

Butane-grown *Pseudomonas butanovora* oxidized butane by a soluble butane monooxygenase through the terminal pathway yielding 1-butanol as the predominant product. Alcohol dehydrogenases (ADHs) involved in butane oxidation in *P. butanovora* were purified and characterized at the biochemical, genetic and physiological levels. Butane-grown *P. butanovora* expressed a type I soluble quinoprotein 1-butanol dehydrogenase (BOH), a soluble type II quinoxinoprotein 1-butanol dehydrogenase (BDH) and an NAD⁺-dependent secondary ADH. Two additional NAD⁺-dependent secondary ADHs were also detected in cells grown on 2-butanol and lactate. BDH was purified to near homogeneity and characterized. BDH is a monomer of 66 kDa consisting of one mole of pyrroloquinoline quinone (PQQ) and 0.25 mole of heme *c* as the prosthetic groups. BOH was partially purified and its deduced amino acid sequence suggests a 67-kDa ADH containing a PQQ as a cofactor. BOH and BDH exhibited high activities and preference towards 1-butanol and fair preference towards butyraldehyde. While BDH could not oxidize 2-butanol, BOH is capable of

2-butanol oxidation and has a broader substrate range than that of BDH. Genes encoding BOH and BDH and their deduced amino acid sequences were identified. BOH and BDH mRNAs and 1-butanol oxidation activity were induced when cells were exposed to butane. Primary C₂ and C₄ alcohols were the most effective inducers for *boh* and *bdh*. Some secondary alcohols, such as 2-butanol, were also inducers for BOH mRNA, but not for BDH mRNA. Insertional inactivation of *boh* or *bdh* affected unfavorably, but did not eliminate, butane utilization in *P. butanovora*. The *P. butanovora* mutant strain with both *boh* and *bdh* genes disrupted was unable to grow on butane and 1-butanol. This result confirmed the involvement of BOH and BDH in butane and 1-butanol metabolism in *P. butanovora*. Roles of BOH and BDH in butane and 1-butanol metabolism were further studied at the physiological level. There are no substantial differences between BOH and BDH in the mRNA expressions in response to three different 1-butanol levels tested and in their abilities to respond to 1-butanol toxicity. Different bioenergetic roles of BOH and BDH in butane and 1-butanol metabolism were suggested. A model of 1-butanol-dependent respiratory systems was proposed where the electrons from 1-butanol oxidation follow a branched electron transport chain. The role of BOH was suggested to function primarily in energy generation because BOH may couple to ubiquinone with the electrons being transported to a cyanide-sensitive terminal oxidase. BDH may be more important in the detoxification of 1-butanol because the electrons from BDH may be transferred to a terminal oxidase system that is less sensitive to cyanide, which is not capable of energy generation.

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May 17, 2002

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Biochemical, Molecular and Physiological Characterization
of 1-Butanol Dehydrogenases of *Pseudomonas butanovora*
in Butane and 1-Butanol Metabolism

by

Alisa S. Vangnai

A THESIS

Submitted to

Oregon State University

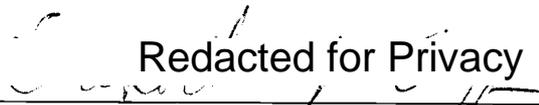
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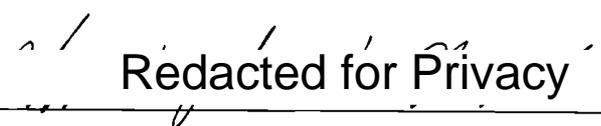
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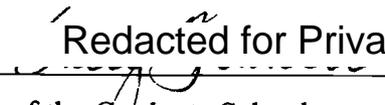
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CONTRIBUTION OF AUTHORS

Dr. Daniel J. Arp contributed to the experimental design and manuscript preparation for chapters 2, 3, 4. Dr. Luis A. Sayavedra-Soto contributed to experimental design and experimental data on the characterization of the *boh::tet* strain mutant presented in chapter 3 and assisted with the preparation of the manuscript from chapter 4. All experiments were financially supported and conducted in the laboratory of Dr. Daniel J. Arp at Oregon State University.

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**BIOCHEMICAL, MOLECULAR AND PHYSIOLOGICAL
CHARACTERIZATION OF 1-BUTANOL DEHYDROGENASES OF
Pseudomonas butanovora IN BUTANE AND 1-BUTANOL METABOLISM**

CHAPTER 1.

INTRODUCTION

1.1 ALKANES IN THE ENVIRONMENT

Natural sources, such as vegetation and microorganisms, are the most important contributors of hydrocarbons in the environment (Manahan, 2000). Hydrocarbons emitted by plants include, for example, ethylene and terpenes. Anaerobic bacteria produce methane in large quantities in the decomposition of organic matter in water, sediments and soil. However, because of human activities, hydrocarbons and derivatives used in fuels and in other industrial applications are generated and released in large quantities into the atmosphere, soil, ground water and marine environment and predominate among organic pollutants (Jirku *et al.*, 2000; Manahan, 2000; National Research Council, 1993).

Alkanes are the major constituents in crude oil and synthetic gasoline (Lyman *et al.*, 1992). They are also among the more stable hydrocarbons in the atmosphere. Straight-chain alkanes with 1 to more than 30 carbon atoms and branched-chain alkanes with 6 or fewer carbon atoms are commonly present in polluted environments. To minimize these hydrocarbon pollutants, there are several treatment methods: physical, chemical, thermal treatments and bioremediation. Bioremediation refers to the use of microbial processes to clean up or to minimize the hazardous pollutants in the environment (Madigan *et al.*, 2000; Manahan, 2000). The biodegradability of alkanes is influenced by 1) their

physical properties, such as solubility in water and vapor pressure, 2) their chemical properties, including molecular structure and the presence of functional groups, and 3) the environmental determinants such as temperature, pH, and oxygen level. Many studies have shown that low-molecular weight *n*-alkanes are readily biodegraded. Longer-chain and branching alkanes or alkanes with an increase in ring structure decrease biodegradability (Blackburn *et al.*, 1993; Huesemann, 1995; Leahy & Colwell, 1990).

1.2 POTENTIAL ADVANTAGES OF ALKANE-OXIDIZING BACTERIA

The definition of cometabolism defined as “the transformation of a non-growth substrate in the obligate presence of a growth substrate or another transformable compound” arose from a study of hydrocarbon oxidation of methane-oxidizing bacteria (Arp *et al.*, 2001; Dalton & Stirling, 1982). This principle has been applied for the bioremediation of chlorinated solvents which cause serious environmental problems through contamination of ground water, drinking water and soil (Arp *et al.*, 2001). Degradation of target compounds occurs because of their fortuitous oxidation by enzymes, which function physiologically to initiate the oxidation of a growth substrate. Methane (Chang & Alvarez-Cohen, 1996; Oldenhuis *et al.*, 1989), propane (Wackett *et al.*, 1989), ammonia (Rasche *et al.*, 1990; Vannelli *et al.*, 1990) and toluene (McClay *et al.*, 1996; Wackett & Gibson, 1988) are examples of growth substrates which support the cometabolism of several chlorinated aliphatic hydrocarbons. Potential advantages of using alkanes, such as propane and butane, as growth substrate for alkane-oxidizing bacteria in the *in situ* bioremediation by aerobic cometabolism is a promising method for remediating contaminated sites because they are highly soluble in

water, inexpensive and readily available. Propane and butane were shown to be effective cometabolic substrate to drive the transformation of chloroform (Hamamura *et al.*, 1997; Kim *et al.*, 1997).

1.3 ALKANE UTILIZATION BY MICROORGANISMS

The utilization of alkanes by microorganisms is initiated by alkane monooxygenase catalyzing the oxidation of alkanes to alcohols. Alkane metabolism and the corresponding monooxygenases can be divided into three groups based on the number of carbon atoms in the alkane substrates: methane (C_1), gaseous short-chain alkanes (C_2 - C_5), and liquid long-chain alkanes (C_6 - C_{20}).

1.3.1 Methane utilization

Methane can be utilized by methanotrophs, which are widespread in natural habitats (Hanson & Hanson, 1996). Methanotrophs play important roles in carbon cycling because they are probably the largest biological sink for methane in aerobic soils (King, 1992; McDonald & Murrell, 1997) and in bioremediation because of their ability to degrade ground water contaminants, including trichloroethylene (Alvarez-Cohen *et al.*, 1992; Arp *et al.* 2001).

1.3.1.1 Methane-utilizing bacteria

Methylotrophic bacteria are gram-negative aerobic bacteria that can utilize one-carbon compounds such as methane, methanol, and methylated compounds as

sources of carbon and energy and assimilate formaldehyde as a major source of cellular carbon (Anthony, 1982; Whittenbury & Dalton, 1981; Whittenbury *et al.*, 1970). Methanotrophs or methane-oxidizing bacteria, a subset of methylotrophs, are unique in their ability to utilize methane as a sole carbon and energy source (Hanson & Hanson, 1996). They are taxonomically classified into three groups (type I, II and X) based on the pathway used for carbon assimilation, morphological differences, and some physiological characteristics (Whittenbury & Dalton, 1981). Type I methanotrophs, including *Methylomonas* and *Methylobacter*, utilize ribulose monophosphate (RuMP) as the primary pathway for formaldehyde assimilation. Type II methanotrophs, including the genera *Methylosinus* and *Methylocystis*, utilize the serine pathway for formaldehyde assimilation. Type X methanotrophs, such as *Methylococcus capsulatus* Bath, were distinguished from type I and type II because they have properties of both types (Bowman *et al.*, 1993; Bowman *et al.*, 1995; Whittenbury & Dalton, 1981; Whittenbury *et al.*, 1970).

1.3.1.2 Methane oxidation

The oxidation of methane by aerobic methanotrophs is initiated by methane monooxygenases (MMOs) by which two reducing equivalents are utilized to split the O-O bond of dioxygen. One of the oxygen atoms is reduced to form H₂O and the other is incorporated into methane to form methanol (Hanson & Hanson, 1996; Lipscomb, 1994). Besides methane being an inducer for MMOs, methanol has been reported for its ability to promote atmospheric methane oxidation in methanol-treated methanotrophic cultures and soils (Benstead *et al.*, 1998).

Two forms of MMOs have been found in methanotrophic bacteria. All methanotrophs are capable of expressing a membrane-bound particulate form of MMO (pMMO) at high copper-biomass ratio (>0.85 to 1 $\mu\text{mol/g}$ cell dry weight)

(Collins *et al.*, 1991; Hanson & Hanson, 1996; Nguyen *et al.*, 1994). On the other hand, a soluble, cytoplasmic form of MMO (sMMO) has been observed only in some methanotrophs: type II (*Methylosinus* and *Methylocystis*), type X (*Methylococcus capsulatus*) and one type I methanotroph (*Methylococcus capsulatus* (Bath)) (Koh *et al.*, 1993) at low copper-biomass ratios (<0.85/g cell dry weight).

1.3.1.3 The soluble methane monooxygenase (sMMO)

sMMO enzymes have been characterized extensively from *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b (Lipscomb, 1994). The sMMO is a non-heme iron-containing enzyme complex consisting of three components. The hydroxylase consists of three subunits of 60, 45 and 20 kDa arranged in an $\alpha_2\beta_2\gamma_2$ configuration. The α subunit contains a non-heme hydroxy-bridged diiron center where methane and oxygen interact to form methanol at the enzyme active site (Murrell *et al.*, 2000). sMMO also contains a component of approximately 16 kDa, Protein B, which has a regulatory role. At low concentration, Protein B converts the hydroxylase from an oxidase to a hydroxylase and stabilizes intermediates necessary for oxygen activation. Saturating amount of Protein B increases the rates of formation of intermediates and accelerates catalysis of methane to methanol by sMMO. The third component, Protein C, is a 39-kDa NADH-dependent, [2Fe-2S]- and FAD-containing reductase that accepts electrons from NADH and transfer them to the diiron sites of the hydroxylase (Lund *et al.*, 1985). sMMO has a broad substrate specificity and can oxidize a wide range of non-growth substrate such as alkanes, alkenes and aromatic compounds. The genes encoding sMMO from several methanotrophs have been cloned and sequenced including those from *Methylococcus capsulatus* (Bath) and

Methylosinus trichosporium OB3b (Murrell, 1994). sMMO genes are clustered on the chromosome of the methanotrophs. *mmoX*, *mmoY* and *mmoZ* encode the α -, β -, and γ -subunits of the hydroxylase, respectively. *mmoB* and *mmoC* code for Protein B and the reductase component. The sMMO genes are highly conserved in all methanotrophs: 55-94% identical in nucleotide sequences and 47-96% identical in amino acid sequences (Murrell *et al.*, 2000).

1.3.1.4 The particulate methane monooxygenase (pMMO)

pMMO was purified and characterized from *Methylosinus trichosporium* OB3b (Takeguchi *et al.*, 1998). The pMMO consists of three subunits of approximately 45, 27 and 23 kDa in a stoichiometry of 1:1:1 (Nguyen *et al.*, 1998; Zahn & DiSpirito, 1996). The active enzyme contains 2 iron and approximately 15 copper atoms per mol. Unlike sMMOs, the pMMO has a relatively narrow substrate specificity, oxidizing alkanes and alkenes of up to five carbons, but not aromatic compounds. The gene encoding pMMO from *Methylococcus capsulatus* (Bath) (Semrau *et al.*, 1995), *Methylosinus trichosporium* OB3b, and *Methylocystis* sp. strain M have been cloned and are clustered on the chromosome. They all have two copies of *pmoCAB* (Murrell *et al.*, 2000).

1.3.2 Long-chain alkane utilization

Long-chain, liquid *n*-alkanes, one of the major constituents in crude oil, can be utilized by both Gram negative and Gram positive bacteria. The oxidation of long-chain alkanes by an alkane hydroxylase has been studied in great detail because it plays an important role in biodegradation of oil-contaminated

environments (Smits *et al.*, 1999). In addition, the alkane hydroxylase system in these organisms is able to carry out a wide range of stereoselective and regioselective oxidation reactions; thus, it is commercially useful as a biocatalyst for the synthesis of pharmaceuticals or other fine chemicals (Smits *et al.*, 1999; Staijen *et al.*, 2000; Witholt *et al.*, 1990).

1.3.2.1 Long-chain alkane-utilizing bacteria

The utilization of long-chain, liquid *n*-alkanes as the sole carbon and energy source has been studied most extensively in Gram negative bacteria, genera *Pseudomonas* and *Acinetobacter*, such as *Pseudomonas oleovorans* (Eggink *et al.*, 1988; van Beilen *et al.*, 1992) and *Acinetobacter* sp strain ADP-1 (Geissdorfer *et al.*, 1995; Ratajczak *et al.*, 1998). The substrate range for *P. oleovorans* ranges from *n*-alkane C₆ to C₁₂. Gram positive bacteria, genera *Rhodococcus*, *Mycobacterium*, *Norcardia*, *Gordona* and *Sphingomonas* have also been reported to be able to utilize long-chain *n*-alkanes; however, little is known about their enzyme systems and their genetics regarding the degradation of long-chain *n*-alkanes.

1.3.2.2 Long-chain alkane oxidation

The *n*-alkane oxidation system in *P. oleovorans* is well characterized and carried out by the alkane hydroxylase (*alk*) system (Baptist *et al.*, 1963; McKenna & Coon, 1970) encoded by the *alkBFGHJKL* and *alkST* genes (Eggink *et al.*, 1988) located on the OCT plasmid (Chakrabarty *et al.*, 1973; Kok *et al.*, 1989). Alkanes are oxidized to corresponding primary alcohols through a terminal oxidation

pathway by a three-component monooxygenase. The hydroxylation reaction is carried out by AlkB, a 41-kDa integral cytoplasmic membrane-associated protein with a diiron cluster. AlkB is usually referred to as an alkane hydroxylase (Shanklin *et al.*, 1997). The others, rubredoxin, a small protein with the iron-sulfur center, and rubredoxin reductase, a 55-kDa flavoprotein, are localized in the cytoplasm and are encoded by *alkG* and *alkT*, respectively. During alkane hydroxylation, rubredoxin reductase delivers reducing equivalents supplied by NADH to membrane hydroxylase via the electron carrier, rubredoxin. *Acinetobacter* sp. strains can degrade *n*-alkanes ranging from C₁₂-C₄₄ as a sole carbon source through several oxidation pathways (Asperger & Kleber, 1991; Asperger *et al.*, 1981; Bajapai *et al.*, 1998). For example, an *n*-alkane oxidation pathway through a dioxygenase reaction was postulated in *Acinetobacter* sp. strain M-1 (Maeng *et al.*, 1996) while two alkane hydroxylase complexes switched by different chain lengths of *n*-alkanes were later described in the same organism (Tani *et al.*, 2001). A cytochrome P-450 monooxygenase was noted in *n*-alkane oxidation in *Acinetobacter calcoaceticus* (Muller *et al.*, 1989) and in *Corynebacterium* 7E1C (Cardini & Jurtshuk, 1970).

1.3.3 Short-chain alkane utilization

The challenge of the utilization of aliphatic alkanes as metabolic substrates is their limited solubility in water. The solubility of aliphatic compounds rapidly decreases with increasing chain length and molecular weight (Watkinson & Morgan, 1990). Gaseous alkanes, such as ethane, propane and *n*-butane have high solubility in water (60-62 ppm) and their transfer rates into water are ~1.5-2.0 times higher than that of methane (solubility of 24 ppm) under the same conditions (Takahashi *et al.*, 1980; Vestel, 1984). Thus, short chain *n*-alkanes are the most

rapidly biodegraded components in both laboratory culture and the natural environment (Kennicutt, 1988; Watkinson & Morgan, 1990).

1.3.3.1 Short-chain *n*-alkane-utilizing bacteria

The ability of bacteria to utilize the short chain, gaseous *n*-alkanes (C₂-C₄) as a sole carbon and energy source is confined mainly to the Gram positive *Corynebacterium-Norcardia-Mycobacterium-Rhodococcus* complex (Ashraf *et al.*, 1994; Perry, 1980). Some Gram negative bacteria, mostly *Pseudomonas* sp. have been described to grow on short chain *n*-alkanes as well (Perry, 1980). The growth substrate range of these short-chain alkane-utilizing bacteria generally extends to liquid alkanes. For example, *Mycobacterium vaccae* JOB5 can utilize alkanes ranging from C₂ to C₄₀ (Murphy & Perry, 1987) and *Nocardioides* sp. strain CF8 can metabolize alkanes ranging from C₂ to C₁₆ (Hamamura & Arp, 2000). In contrast, methanotrophs and long-chain alkane utilizers generally do not grow on short-chain gaseous alkanes (Baptist *et al.*, 1963).

1.3.3.2 *Pseudomonas butanovora*

Pseudomonas butanovora is a Gram negative, rod-shaped bacterium. It was isolated from activated sludge from an oil refining plant by using *n*-butane as the energy source (Takahashi *et al.*, 1980). Based on morphological, physiological and biochemical characteristics, this organism was grouped in the genus *Pseudomonas* (Takahashi *et al.*, 1980). However, the sequence of the 16S ribosomal DNA gene is most similar to that of members of the genus *Azoarcus* and *Thauera* (Anzai *et al.*, 2000). This genus is characterized by members that degrade aromatic compounds

(Krieger *et al.*, 1999; Philipp & Schink, 1998) under anaerobic conditions and other members that are plant epiphytes with the ability to fix nitrogen (Reinhold-Hurek *et al.*, 1993). *P. butanovora* can utilize a variety of organic compounds as the growth substrate: C₂-C₉ *n*-alkanes, the corresponding primary alcohols, carboxylic acids and some polyvalent alcohols, but not alkenes, sugars or C₁ compounds (Takahashi, 1980; Takahashi *et al.*, 1980). Butane-grown *P. butanovora* can degrade several chlorinated aliphatic hydrocarbons (Hamamura *et al.*, 1997), which indicates the potential of butane-grown *P. butanovora* in the bioremediation of sites contaminated with solvents.

1.3.3.3 Short-chain *n*-alkane oxidation

Short-chain *n*-alkanes are oxidized by bacterial monooxygenase to alcohols via a terminal, a subterminal and the mixture of both pathways (Arp, 1999; Ashraf *et al.*, 1994; Phillips & Perry, 1974). The pathways for metabolism of gaseous *n*-alkanes (C₂-C₄) by some of these organisms have been investigated by several techniques. In all cases, metabolism is initiated by a monooxygenase (Ashraf *et al.*, 1994; Hamamura & Arp, 2000; Hamamura *et al.*, 1999; Perry, 1980; Stephens & Dalton, 1986; Woods & Murrell, 1989).

The presence of propane monooxygenase (PMO) has been shown in *Rhodococcus rhodochrous* PNKb1 but the purification and characterization of the enzyme were not possible because of its unstable nature (Woods & Murrell, 1989).

Butane monooxygenases (BMO) have been studied in two Gram positive bacteria, *Nocardioides* sp. strain CF8 and *Mycobacterium vaccae* JOB5 and a Gram negative bacteria, *P. butanovora* (Hamamura *et al.*, 1999). All three strains of bacteria oxidize butane at similar rates, exhibit a strong affinity for butane, and their butane oxidation activities are inhibited by acetylene, a mechanism-based

inactivator of several monooxygenases. The presence of three distinct BMOs in these bacteria was indicated by the different responses to the known monooxygenase inactivator, ethylene, and the inhibitor, allythiourea (ATU). Ethylene oxide, the product of ethylene oxidation, irreversibly inactivated butane oxidation by *P. butanovora* but not by *M. vaccae* or *Nocardioides* sp. strain CF8. In contrast, butane oxidation by only *Nocardioides* sp. strain CF8 was strongly inhibited by ATU, a copper selective chelator which reversibly inhibits copper-containing pMMO and ammonia monooxygenase (AMO) (Bedard & Knowles, 1989). The inhibition and inactivation profiles imply the presence of different prosthetic groups in BMO among three butane-oxidizing bacteria (Hamamura *et al.*, 1999).

The pathway of butane metabolism in *P. butanovora* has been determined to follow the terminal oxidation pathway, that is butane → 1-butanol → butyraldehyde → butyrate (Arp, 1999). Each intermediate in the pathway accumulated in the presence of appropriate inhibitors. The oxidation of butane is initiated by the action of a monooxygenase and up to 86% of butane oxidized is accounted for as 1-butanol produced, suggesting that the terminal pathway is predominant. Although butane-grown cells consumed 2-butanol, 2-butanol production can not be detected even in the presence of appropriate inhibitors for 2-butanol consumption. Butane oxidation is not repressed by the metabolites of butane but is repressed by lactate (Sayavedra-Soto *et al.*, 2001).

The butane monooxygenase (sBMO) from *P. butanovora* is soluble and has high similarity to soluble methane monooxygenase (sMMO), for example 64% identity of the hydroxylase α subunits. Like sMMO, sBMO consists of a hydroxylase component, a reductase component and a small coupling protein. The theoretical molecular mass of each component is estimated from the deduced amino acid sequence: a hydroxylase α subunit (530 amino acid residues, 60.7 kDa), a hydroxylase β subunit (391 amino acid residues, 45 kDa), a coupling protein (137

amino acid residues, 15 kDa), a hydroxylase γ subunit (168 amino acid residues, 19 kDa), a reductase (364 amino acid residues, 39.8 kDa) (Sluis & Arp, unpublished). The genes encoding sBMO had also been identified. The gene organization is, in order and same orientation, *bmoX*, *bmoY*, *bmoB*, *bmoZ*, *bmoD* and *bmoC* which code for the hydroxylase α subunit, the hydroxylase β subunit, a regulatory protein, the hydroxylase γ subunit, a putative assembly/OrfY protein, and a reductase, respectively.

1.4 MICROBIAL ALCOHOL DEHYDROGENASES

Alcohol dehydrogenases (ADHs) or alcohol oxidoreductases are involved in a wide range of microbial metabolisms and have a wide variety of substrate specificities including alcohols, the corresponding aldehydes and ketones. Some of these enzymes are involved in the production of alcoholic beverages, of vinegar, and of industrial solvents. Others are important in the degradation of naturally occurring and xenobiotic aromatic compounds (Reid & Fewson, 1994). The conversions of alcohols, aldehydes and ketones catalyzed by ADHs use a variety of different electron acceptors; thus, ADHs can be divided into three groups: 1) the NAD(P)-dependent ADHs, 2) the NAD(P)-independent ADHs, and 3) FAD-dependent alcohol oxidases.

1) The NAD(P)-dependent ADHs. The NAD(P)-dependent ADHs found in animals, plants and microorganisms show certain structural and functional similarities (Jornvall *et al.*, 1987). These enzymes catalyze the reaction:



The NAD(P)-dependent ADH superfamily can be divided into three groups; Group I zinc-dependent long chain ADHs (containing approximately 350 residues per subunit); Group II zinc-dependent short chain ADHs (containing approximately

250 residues per subunit); Group III iron-activated ADHs. Of these three NAD(P)-dependent groups, the group I ADHs are the most studied (Sun & Plapp, 1992; Yokoyama *et al.*, 1990).

The group I NAD(P)-dependent ADHs contain highly divergent members including dimeric mammalian/plant ADHs (such as human ADHs and horse liver ADH), bacterial ADHs, tetrameric yeast ADHs, and *E. coli* threonine dehydrogenase. These proteins are grouped together because of their sequence identities and/or structure-function similarities, although the amino acid sequence alignment of 47 members of this group showed that there are only nine amino acid residues conserved throughout all members and they all have structural roles (Sun & Plapp, 1992). Benzyl ADH encoded by TOL plasmid (pWVO) of *Pseudomonas putida* (Shaw *et al.*, 1993) and the chromosomally encoded benzyl ADH of *Acinetobacter calcoaceticus* (Chalmers *et al.*, 1991; Gillooly *et al.*, 1998) are members of this group.

Group II, the zinc-dependent short chain ADH family, is not as well characterized as group I. The members of this group include ADH from *Drosophila* spp. and from some prokaryotes, for example, *cis*-toluene dihydrodiol dehydrogenase of *P. putida* and biphenyl dihydrodiol dehydrogenase of *Pseudomonas* sp. (Reid & Fewson, 1994). Group III, iron-activated ADHs that have been identified on the basis of their primary structures and subunit size (approximately 385 residues) so far are microbial ADHs. The enzymes in this group mainly involve in the production of alcohols in microbial fermentation processes, for example, ADH of *Zymomonas mobilis*, and *Clostridium acetobutylicum* (Reid & Fewson, 1994).

2) The NAD(P)-independent ADHs. The NAD(P)-independent ADHs contain either pyrroloquinoline quinone (PQQ) or cofactor F₄₂₀ as a cofactor. ADHs in this group catalyze the reaction: Alcohol + 2X_(ox) ⇌ Aldehyde + 2X_(red) + 2H⁺ where

$2X_{(ox)}$ and $2X_{(red)}$ are representative of the oxidized and reduced form of the electron acceptor. Besides methanol dehydrogenase, the PQQ-containing ADHs can be divided into three types (type I, II and III). Their biochemical characteristics and genetics will be discussed in detail below. The F_{420} -dependent ADHs are found in methanogenic bacteria such as *Methanogenium liminatans* and *Methanobacterium palustre* (Bleicher & Winter, 1991).

3) FAD-dependent alcohol oxidases. FAD-dependent alcohol oxidases catalyze irreversible oxidation of alcohols. Enzymes in this group are mainly found in the methylotrophic yeasts and filamentous fungi catalyzing the oxidation of methanol to formaldehyde. They have been characterized as flavoproteins containing one noncovalently-bound FAD prosthetic group per subunit. They are different from other ADHs in that they oxidize alcohols to aldehydes irreversibly with the concurrent production of hydrogen peroxide (Veenhuis *et al.*, 1983).

1.5 ALCOHOL OXIDATION IN ALKANE AND ALCOHOL METABOLISMS

The oxidation of alkane yields alcohol(s) which is further oxidized by alcohol dehydrogenase(s). Alcohol dehydrogenases are probably the best-characterized enzymes of gaseous alkane degradation pathways. In some cases, the detection of alcohol dehydrogenases leads to understanding of the metabolic sequence of the alkane metabolism (Vestel, 1984). ADHs involved in alkane metabolism have been identified with different prosthetic groups, including metals, pyrroloquinoline quinone (PQQ), flavin and heme. Cytochromes and NAD^+ have also been shown to be electron acceptors for these enzymes.

1.5.1 NAD⁺ dependent alcohol dehydrogenases in *n*-alkane metabolism.

The NAD⁺-dependent ADHs involved in alkane metabolisms have been reported in both Gram positive and Gram negative bacteria. For example, in *M. vaccae* JOB5 grown on propane or 2-propanol, an inducible NAD⁺-dependent 2-propanol dehydrogenase was detected suggesting a subterminal pathway for propane oxidation (Coleman & Perry, 1985). In *Rhodococcus rhodochrous* PNKb1 (Ashraf *et al.*, 1994; Ashraf & Murrell, 1992) and *P. fluorescens* NRRL B-1244 (Ashraf & Murrell, 1992; Hou *et al.*, 1983), separate NAD⁺-dependent ADHs are involved in the oxidation of primary and secondary alcohols (1-propanol and 2-propanol) suggesting the presence of both terminal and subterminal oxidation pathways of propane. In *P. oleovorans*, 1-octanol is oxidized to octanal by a flavoprotein dehydrogenase (van Beilen *et al.*, 1992).

1.5.2 Pyrroloquinoline quinone (PQQ)

Pyrroloquinoline quinone (PQQ) was first isolated as a prosthetic group for glucose dehydrogenase (Hauge, 1964) and methanol dehydrogenase (Anthony, 2001; Anthony & Zatman, 1967). PQQ (4,5-dihydro-4,5-dioxo-1*H*-pyrrolo[2,3-*f*]quinoline-2,7,9-tricarboxylic acid), originally called methoxatin, is highly soluble, heat-stable and non-covalently binds to proteins as a redox prosthetic group facilitating the oxidation of alcohols and aldose sugars in the periplasm of Gram negative bacteria (Anthony, 2001; Stites *et al.*, 2000). The key feature of PQQ structure is the *ortho*-quinone at the C4 and C5 positions of the quinoline ring (Fig. 1.1), which becomes reduced to the PQQ hydroquinone (or called quinol) (PQQH₂) during catalysis (Duine, 1989; Stites *et al.*, 2000).

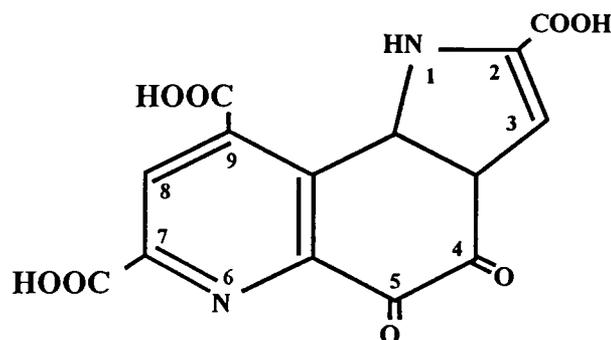


Figure 1.1 Structure of pyrroloquinoline quinone (PQQ)

1.5.2.1 PQQ biosynthesis in bacteria

Numerous bacteria, including methylotrophic bacteria, *Pseudomonas* sp., *Acetobacter* sp., and *Gluconobacter* sp., are capable of PQQ synthesis (Duine *et al.*, 1990). To date, the best evidence suggests that PQQ is synthesized from the condensation of L-glutamate and L-tyrosine; however, the biochemical steps of PQQ biosynthesis are still unknown (Anthony, 2001; Houck *et al.*, 1988; Stites *et al.*, 2000; Van Kleef & Duine, 1988). Although the pathway for PQQ biosynthesis remains to be resolved, seven genes in two separate clusters required for PQQ biosynthesis have been cloned from several organisms (e.g. *Acinetobacter calcoaceticus* (Goosen *et al.*, 1989), *Pseudomonas fluorescens* CHAO (Schnider *et al.*, 1995), *Methylobacterium extorquens* AM1 (Morris *et al.*, 1995; Toyama *et al.*, 1997)).

1.5.2.2 Importance of PQQ to microorganisms

PQQ has been shown to have a growth-stimulating property for some microorganisms such as *Gluconobacter oxydans* IFO 3287 (Ameyama & Adachi, 1986), *Comamonas testosteroni* (Groen *et al.*, 1986), and *Pseudomonas* sp. VM15C (Shimao *et al.*, 1984), as well as for plants and animal cells in culture (Ameyama & Adachi, 1986). Under certain growth conditions, PQQ can be excreted from some bacteria, such as *Pseudomonas aeruginosa* (Ameyama & Adachi, 1987), *Methylobacterium organophilum* XX (Ameyama *et al.*, 1988). It is also a chemostatic attractant for some microorganisms such as *E. coli*, which produces apo-glucose dehydrogenase (GDH) because of a defect in gene coding for PQQ biosynthesis.

1.5.2.3 PQQ as a prosthetic group of bacterial dehydrogenases

Bacterial dehydrogenases that have PQQ as the prosthetic group are called quinoproteins or quinoprotein dehydrogenases. PQQ-containing enzymes catalyze the oxidation of alcohols (alcohol dehydrogenases) and glucose (glucose dehydrogenase). There are three types of PQQ-containing ADHs (type I, II and III), and methanol dehydrogenase. There are two types of glucose dehydrogenase (a soluble form and a membrane-associated form) (Anthony, 2001; Duine, 1989).

1.5.3 Methanol dehydrogenase

Methanol dehydrogenase (MDH) is the second enzyme in the methane oxidation pathway. Methanol dehydrogenase oxidizes methanol to formaldehyde

during growth of bacteria on methane or methanol (Anthony, 1986). Methanol dehydrogenase of methylotrophic bacteria is the most fully described PQQ-containing alcohol dehydrogenase.

1.5.3.1 Biochemistry of MDH

Methanol dehydrogenase is a soluble periplasmic enzyme having an $\alpha_2\beta_2$ tetrameric structure; each α subunit (average size of 60 kDa, and 66 kDa in *Methylobacterium extorquens*) contains one molecule of PQQ and one Ca^{2+} ion. The β subunit is a small protein subunit (8.5 kDa in *Methylobacterium extorquens*) of unknown function (Anthony, 1986). MDH oxidizes a wide range of primary alcohols with high affinity to methanol (K_m for methanol is 5-20 μM) (Goodwin & Anthony, 1998). The affinity of MDH decreases with increasing size of the primary alcohols. Secondary alcohols are rarely oxidized (Anthony, 1986; Goodwin & Anthony, 1998). MDH also has ability to oxidize formaldehyde to formate (Hiptinstall & Quayle, 1969; Ladner & Zatman, 1969). MDH assayed *in vitro* with phenazine methosulfate (PMS) as an artificial electron acceptor at high pH value requires an activator, ammonium salts or methylamine. The requirement of ammonium or amine activation in MDH activity assay is suggested to be involved in the re-oxidation of the reduced enzyme and not for the reduction of enzyme by its substrate (Duine & Frank, 1981; Duine & Frank, 1980). The mechanism of ammonium activation is still unclear (Anthony, 1986; Anthony, 1998). Recently, the X-ray structures of MDHs from *Methylophilus methylotrophus* W3A1 and of *Methylobacterium extorquens* determined at 1.94 Å have been reported (Afolabi *et al.*, 2001; Ghosh *et al.*, 1995; Xia *et al.*, 1996).

1.5.3.2 Genetics of MDH

Studies on the genetics of methanol oxidation in *Methylobacterium* strains reveals that at least 26 genes are required in this process (Amaratunga *et al.*, 1997; Morris *et al.*, 1995; Springer *et al.*, 1998; Springer *et al.*, 1995). These methanol oxidation genes are mapped to four loci on the *M. extorquens* AM1 chromosome. The first locus contains a cluster of 12 known genes: *mxafJGIRSACKLDB* with an additional gene (*mxaw*) adjacent to *mxaf* and divergently transcribed (Morris & Lidstrom, 1992; Xu *et al.*, 1993). *mxaf* and *mxal* encode the structural proteins, the large (α) and small (β) subunits, of MDH, respectively. *mxag* encodes cytochrome c_L , the primary electron acceptor for MDH. *mxackl* are required for insertion of calcium into MDH and *mxar* is involved in the regulation of formation of active MDH. The functions of *mxaj* and *mxaw* remain unknown (Springer *et al.*, 1998). *mxbdm*, *mxqce*, and *mxab* encode regulatory proteins of the response regulatory-sensor kinase family. They are required for transcription of *mxaf* in both *M. extorquens* AM1 and *M. organophilum* XX (Springer *et al.*, 1998; Springer *et al.*, 1997; Xu *et al.*, 1995). To date, the *mxaf* genes from four methylotrophic bacteria strains, *M. extorquens* AM1 (Anderson *et al.*, 1990), *M. organophilum* XX (Machlin & Hanson, 1988), *Paracoccus denitrificans* PD1207 (Harms *et al.*, 1987), and *Methylophilus methylotrophus* W3A1 (Xia *et al.*, 1996) have been cloned and sequenced. The *mxaf* genes of two type I methanotrophs, *Methylococcus capsulatus* (Bath) and *Methylomicrobium album* BG8 have been cloned; however, no complete sequences have been reported (McDonald & Murrell, 1997).

The use of *mxaf* as a functional gene probe to identify methanotrophs has been reported (McDonald & Murrell, 1997). While the gene encoding *mxaf* can be found in all methanotrophs and also in gram negative methylotrophs, the sMMO gene is not universal to all methanotrophs and is found predominantly in the genera *Methylosinus* and *Methylococcus* (Stainthorpe *et al.*, 1990)

1.5.3.3 Electron transport system of MDH

When MDH catalyzes the oxidation of methanol, the electrons are transferred from the reduced PQQ to its specific physiological electron acceptor, cytochrome c_L , in the periplasm (Day & Anthony, 1990) or to branches of cytochrome c_{550} and cytochrome c_{553i} in *Paracoccus* (Baker *et al.*, 1998; Goodwin & Anthony, 1998). Cytochrome c_L is a novel class of c -type cytochrome because it does not have similarity to the conserved amino acid sequences found in other c -type cytochrome (Anthony, 1992). The cytochrome c_L in methylotrophs is not a substrate for the terminal oxidase because its function is to mediate electrons between MDH and a subsequent electron acceptor. Cytochrome c_L is oxidized by cytochrome c_H (H stands for the higher isoelectric point of cytochrome c_H relative to cytochrome c_L) in most methylotrophs (Afolabi *et al.*, 2001; Anthony, 1992; Anthony, 1992), but by a blue copper protein, azurin, in organism strain 4025 (Auton & Anthony, 1989). Cytochrome c_H is a typical Class I soluble c -type cytochrome determined from its conserved amino acid sequence. The function of cytochrome c_H is to mediate electrons from cytochrome c_L or cytochrome bc_1 complex to the terminal oxidase(s), cytochrome aa_3 or cytochrome co , depending on the type of methylotrophs and the growth conditions. This electron transport chain bypasses the low potential ubiquinone/cytochrome bc_1 complex of the chain. Therefore, the first step of methanol oxidation is likely to yield only one ATP molecule (or less) (Goodwin & Anthony, 1998).

1.5.4 Type I soluble quinoprotein alcohol dehydrogenases

Type I quinoproteins are mostly described as ethanol dehydrogenases (EDHs) in *Pseudomonas aeruginosa* (Gorisch & Rupp, 1989; Groen *et al.*, 1984),

Pseudomonas putida (Toyama *et al.*, 1995) and *Rhodopseudomonas* (Bamforth & Quayle, 1978). Besides EDHs, a butanol dehydrogenase in *P. butanovora* grown on butane or 1-butanol has been recently reported as a quinoprotein having high activity and preference towards 1-butanol.

1.5.4.1 Biochemistry of type I quinoproteins

Type I quinoproteins are soluble periplasmic enzymes having a noncovalently-bound PQQ as a prosthetic group. Different structures of EDHs have been reported: $\alpha_2\beta_2$ tetrameric structure in *P. aeruginosa* (Schrover *et al.*, 1993), α_2 dimeric structure in *P. putida* HK5, with the average size of α subunit of ~60-65 kDa (Toyama *et al.*, 1995), and a monomeric protein of 101 kDa in *P. aeruginosa* strain LMD 80.53 (Groen *et al.*, 1984). EDH is able to oxidize a wide range of alcohol substrates including secondary alcohols. It has high affinity for ethanol ($K_m \sim 15 \mu\text{M}$) and low affinity for methanol (K_m is about 1000 times higher than that for ethanol) (Goodwin & Anthony, 1998). Similar to MDH, EDH has a high pH optimum and requires ammonium or alkylamines as an activator in the dye-linked assay system. The X-ray structure of the homodimeric EDH of *P. aeruginosa* has been recently solved at 2.6 Å resolution (Keitel *et al.*, 2000).

1.5.4.2 Genetics of type I quinoproteins

To date, only the genes encoding EDH from *P. aeruginosa* (Diehl *et al.*, 1998) and for 1-butanol dehydrogenase-1 (BOH) from *P. butanovora* (Vangnai *et al.*, 2002) have been sequenced. EDH and BOH amino acid sequences share 70% identity and both sequences show 21% and 33% identities to the α subunit of

MDH, respectively. The gene organization in *P. aeruginosa* shows that five genes encoding components of the quinoprotein ethanol oxidation system form a cluster. The *exaA* coding for EDH is found upstream and in reverse orientation of *exaB*, *exaC*, *pqqA*, and *pqqB* genes, which encode for cytochrome *c₅₅₀*, acetaldehyde dehydrogenase, and genes involved in the biosynthesis of the prosthetic group PQQ, respectively (Schobert & Gorisch, 1999). The gene organization in *P. butanovora* is, in order, *cyc_b*, *bld* (in reverse orientation), *bohR*, and *boh* which codes for a putative cytochrome *b₅₆₁*, an NAD⁺-dependent aldehyde dehydrogenase, a transcriptional regulator, and 1-butanol dehydrogenase-1 (BOH), respectively (Vangnai *et al.*, 2002).

1.5.4.3 Electron transport system of type I quinoproteins

The electron transport systems for soluble periplasmic type I ADHs are thought to be similar to those for methanol oxidation (Goodwin & Anthony, 1998). The soluble quinoprotein ADH from *P. aeruginosa* grown on ethanol reacts rapidly with a small, 14.5-kDa, cytochrome *c₅₅₀* (or called cytochrome *c_{EDH}*). However, there is no similarity between amino acid sequences of cytochrome *c_{EDH}* and cytochrome *c_L* (Schrover *et al.*, 1993). In contrast, the sequence alignment of cytochrome *c_{EDH}* shows 31-33% identity to the C-terminal heme domain of the membrane-bound type III quinohemoprotein ADHs of acetic acid bacteria (Schrover *et al.*, 1993) and to approximately 26% or less to heme domain of type II quinohemoprotein ADH of *Comamonas testosteroni* (Stoorvogel *et al.*, 1996). Cytochrome *c_{EDH}* transfers electrons to *co*-type cytochrome oxidase (Matsushita *et al.*, 1982; Reichmann & Gorisch, 1993). However, it is unclear which membrane component mediates electron flow from cytochrome *c_{EDH}* to the oxidase (Reichmann & Gorisch, 1993).

1.5.5 Type II soluble quinohemoprotein alcohol dehydrogenases

Type II quinohemoproteins are soluble periplasmic ADHs containing two prosthetic groups--one molecule of PQQ and a single heme *c*. Type II ADHs have been purified as the apoenzyme, lacking PQQ, from two organisms: alkaloid-degrading *Pseudomonas* (Hopper *et al.*, 1991) and ethanol-grown *C. testosteroni* (De Jong *et al.*, 1995; Groen *et al.*, 1986), and as the active holoenzyme from these following organisms: *P. putida* HK5 grown on 1-butanol (ADH-IIB) or glycerol (ADH-IIG) (Toyama *et al.*, 1995), *Ralstonia eutropha* grown on tetrahydrofurfuryl alcohol (Zarnt *et al.*, 1997), and *P. butanovora* grown on butane or 1-butanol (Vangnai & Arp, 2001).

1.5.5.1 Biochemistry of type II quinohemoproteins

Type II ADHs are monomeric soluble quinohemoproteins of ~67-75 kDa which contain a PQQ and a usually covalently bound heme *c*. Type II ADH has a wide specificity for primary alcohols and secondary alcohols, but it is unable to oxidize methanol. It also oxidizes aldehydes and large molecules such as steroid as substrates. It has been also exploited for the oxidation of industrially important precursor molecules (synthons) (Geerlof *et al.*, 1994; Goodwin & Anthony, 1998).

The presence of PQQ induces a protein conformational change, and a reorientation of the methionine ligand of heme *c*, therefore resulting in an increase in midpoint redox potential of the heme *c*. Although the interactions between the two cofactors remain unsolved, it is clear that in alcohol oxidation electrons are transferred from the reduced form of PQQ to heme *c* (mid point redox potential of 140 mV), then to an external electron acceptor (De Jong *et al.*, 1995b). Because of the involvement of heme *c* in electron flow, type II ADHs can also be assayed by

using ferricyanide, in addition to the dye-linked assay system (Goodwin & Anthony, 1998). The X-ray structure of a soluble monomeric ADH-IIB in *P. putida* HK5 has been studied at 1.9 Å (Chen *et al.*, 1999).

1.5.5.2 Genetics of type II quinohemoprotein

The genes encoding for type II quinohemoprotein from *Comamonas testosteroni* (Stoorvogel *et al.*, 1996), *Ralstonia eutropha* (Zarnt *et al.*, 2001), and *P. butanovora* (Vangnai *et al.*, 2002) have been sequenced. The amino acid sequence of EDH in *C. testosteroni* and of tetrahydrofurfuryl ADH in *R. eutropha* share 70% identity to the amino acid sequence of 1-butanol dehydrogenase-2 (BDH) in *P. butanovora*. The gene organization in *P. butanovora* shows, in order, a gene coding for a transcriptional regulator (*bdhR*) in reverse orientation, a sequence of unknown function (*orf1*), a 1-butanol dehydrogenase-2 (*bdh*), and a NAD⁺-dependent aldehyde dehydrogenase (*bad*). In contrast, the sequences located upstream and downstream from the *tfaA* gene coding for tetrahydrofurfuryl ADH in *R. eutropha* did not reveal any relevant genes which might be involved in PQQ synthesis as was found in *P. aeruginosa* (for type I ADH) or in further oxidation of aldehyde, the product of alcohol oxidation, as was found in *P. butanovora*.

1.5.5.3 Electron transport system of type II quinoproteins

The physiological electron acceptor in type II quinohemoprotein has not been well studied. To date, there are studies on alcohol oxidation dependent-electron transport systems only from *P. putida* HK5 (Matsushita *et al.*, 1999)

and *P. butanovora* (this study). In 1-butanol-grown *P. putida* HK5, the electron transport system was examined *in vivo* and *in vitro* (Matsushita *et al.*, 2000). Two different ADH-electron transport branches were proposed. A 17.5-kDa copper-containing protein, azurin, was purified from the periplasm of 1-butanol-grown *P. putida* HK5 and was shown to mediate electron transfer from its quinohemoprotein (ADHIIB) to a cyanide-sensitive terminal oxidase (Matsushita *et al.*, 1999) which is likely to be a cytochrome *bc₁-cbb₃* (Matsushita *et al.*, 2000). In addition, a soluble *c*-type cytochrome was proposed to be an electron acceptor in an azurin-independent electron transport system which then mediated electrons to an alternative cyanide-insensitive oxidase. Ubiquinone was also proposed to be another electron acceptor which transferred electrons to both the cyanide-sensitive oxidase and the alternative cyanide-insensitive oxidase (Matsushita *et al.*, 2000).

1.5.6 Type III membrane-associated quinohemoprotein alcohol dehydrogenases

The type III ADH is a membrane-associated quinohemoprotein-cytochrome *c* complex. This enzyme catalyzes the oxidation of ethanol to acetic acid and has only been described in the acetic acid bacteria, *Acetobacter* and *Gluconobacter* (Matsushita *et al.*, 1994).

1.5.6.1 Biochemistry of type III membrane-associated quinohemoproteins

The type III quinohemoprotein is distinguished from other quinoproteins in having three subunits and in being tightly bound to the periplasmic membrane. Subunit I (72-80 kDa) is a quinohemoprotein similar to the soluble (type II) quinohemoprotein ADH, in that it has a single molecule of PQQ and a single heme *c*. Subunit II (48-53 kDa) consists of three heme *c* (Matsushita *et al.*, 1996). Most of these enzymes from acetic acid bacteria have a third subunit, subunit III of 14-17 kDa, but it is absent from *Acetobacter polyoxogenes* (Tayama *et al.*, 1989). It was proven later that the third subunit has no role for ADH activity (Kondo & Horinouchi, 1997). Dissociation of ADH from *Gluconobacter suboxydans* into its subunits and reconstitution indicates that the ethanol oxidation occurs in subunit I/III, while the ubiquinone reductase activity is localized in subunit II (Matsushita *et al.*, 1996).

The function of type III quinohemoprotein ADH together with the membrane-bound aldehyde dehydrogenase is to oxidize ethanol to acetic acid in vinegar production. Its substrate specificity is relatively restricted compared to other quinoprotein ADHs. It oxidizes primary alcohols ranging from C₂-C₆ and has some activity towards formaldehyde and acetaldehyde, but it does not oxidize methanol and secondary alcohols (Goodwin & Anthony, 1998; Matsushita *et al.*, 1994).

1.5.6.2 Genetics of type III membrane-associated quinohemoproteins

Genes encoding subunits I and II in type III membrane-associated quinohemoproteins have been cloned and sequenced in *Acetobacter aceti* (Inoue *et al.*, 1989), *Acetobacter polyoxogenes* (Takeda & Shimiza, 1991), and

Glucobacter suboxydans (Kondo & Horinouchi, 1997; Takeda & Shimiza, 1991). The sequence of subunit I and II each has a leading sequence consisting of 23-35 amino acid residues at the N-terminus, which is consistent with the notion that type III ADH is located on the periplasmic side of the cytoplasmic membrane. The sequence of subunit I shows some similarity to sequences of methanol dehydrogenase in *Paracoccus denitrificans*, *Methylobacterium extorquens* and *Methylobacterium organophilum* (Matsushita *et al.*, 1994).

1.5.6.3 Electron transport system of type III membrane-associated quinohemoproteins

The electron transport systems relating to alcohol oxidation of type III membrane-associated quinohemoproteins have been studied in acetic acid bacteria: *Glucobacter suboxydans*, *Acetobacter aceti*, and *Acetobacter methanolicus*. The electron acceptor for type III ADHs is ubiquinone (Anthony, 1998); however, the respiratory chains are different depending on the types of organisms and the growth conditions. The respiratory chain of *G. suboxydans* branches at ubiquinone, with a cyanide-sensitive cytochrome *o*(*bo*)-oxidase and a cyanide-insensitive bypass oxidase which may contain cytochrome *c*₅₅₃ as one component (Matsushita *et al.*, 1994). The heme *c* in subunit I and two of the three heme *c* moieties in subunit II are involved in the intramolecular electron transport of ADH to ubiquinone, while the function of the remaining heme *c* in subunit II is still unclear (Matsushita *et al.*, 1996). The respiratory chain of *A. aceti* is constituted of the ADH, ubiquinone, and the terminal oxidase. Unlike the respiratory chain in *G. suboxydans*, the respiratory chain in *A. aceti* does not have a cyanide-insensitive bypass oxidase. The alcohol-dependent respiratory chain of *A. aceti* branches at ubiquinone with a cyanide-sensitive cytochrome *a*₁(*ba*)-oxidase when grown in shaking culture and a cyanide-sensitive cytochrome *o*(*bo*)-oxidase when grown in static culture (Matsushita *et*

al., 1992). In *A. methanolicus*, the only acetic acid bacterium that has ability to utilize methanol (Loffhagen & Babel, 1984; Matsushita *et al.*, 1994), two independent cyanide-sensitive respiratory chains were observed. The electron transport chain for methanol oxidation is operated through soluble cytochrome(s) *c* to cytochrome *c*-oxidase, while that in ethanol oxidation is carried out by ubiquinone to cytochrome *o*-ubiquinone oxidase (Matsushita *et al.*, 1994).

1.6 SUMMARY

The alkane oxidation pathways have been studied in a wide range of microorganisms. Among the three groups of alkane oxidation, the pathway of methane oxidation in methanotrophs is the best studied. The metabolic pathway of gaseous *n*-alkanes is the least studied. Butane-oxidizing *Pseudomonas butanovora* serves as the representative of short-chain gaseous *n*-alkane oxidizers. In this organism, butane is oxidized by butane monooxygenase through the terminal pathway mainly producing 1-butanol. The oxidation of alcohol(s) by alcohol dehydrogenase(s) is the second step in the pathway. Since butane-grown *P. butanovora* can consume 1-butanol and 2-butanol, the alcohol oxidation pathway(s) and the substrate specificity of ADHs induced in butane-grown *P. butanovora* have been questioned. Therefore, this dissertation focused on characterization of ADHs involved in the butane oxidation pathway of the butane-oxidizer, *P. butanovora*. Chapter 2 describes the purification, and the biochemical and kinetic characterizations of an inducible 1-butanol dehydrogenase quinohemoprotein involved in the butane oxidation. Chapter 3 describes the characterization of the genes encoding two 1-butanol dehydrogenases, a quinoprotein (BOH) and a quinohemoprotein (BDH). The inactivation of genes encoding both enzymes confirmed their essential involvement in butane metabolism in *P. butanovora*.

Chapter 4 extends the characterization of BOH and BDH at a physiological level. Roles of both enzymes in the butane and 1-butanol metabolism pathway are elucidated. The study of electron transport system(s) through BOH and BDH may also provide more information on the respiratory system(s) of type I and type II quinoproteins which, to date, is still understudied.

CHAPTER 2 .

AN INDUCIBLE 1-BUTANOL DEHYDROGENASE,
A QUINOHEMOPROTEIN,
IS INVOLVED IN THE OXIDATION OF BUTANE
BY *Pseudomonas butanovora*

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2.1. ABSTRACT

Butane-grown *Pseudomonas butanovora* expressed two soluble alcohol dehydrogenases (ADHs), an NAD⁺-dependent secondary ADH and an NAD⁺-independent primary ADH. Two additional NAD⁺-dependent secondary ADHs could be detected when cells were grown on 2-butanol and lactate. The inducible NAD⁺-independent 1-butanol dehydrogenase (BDH) of butane-grown cells was primarily responsible for 1-butanol oxidation in the butane metabolism pathway. BDH was purified to near homogeneity and identified as a quinohemoprotein, containing, per mole of enzyme, one mole of PQQ and 0.25 mole of heme *c* as prosthetic groups. BDH was synthesized as a monomer of approximately 66 kDa. It has a broad substrate range, including primary alcohols, secondary alcohols, aldehydes, C₄ diols, and aromatic alcohols. It exhibited the lowest K_m (7 ± 1 μM) and highest k_{cat}/K_m (7.2 × 10⁵ M⁻¹sec⁻¹) value towards 1-butanol. BDH exhibited ferricyanide-dependent ADH activity. Calcium ion (up to 10 mM) increased BDH activity substantially. Two BDH internal amino acid sequences showed 73% and 62% identity and 83% and 66% similarity, respectively, when compared with an amino acid sequence of ethanol dehydrogenase from *Comamonas testosteroni*. The presence of the inducible BDH and secondary ADH may indicate that the terminal and subterminal oxidation pathways are involved in butane degradation of butane-grown *P. butanovora*.

2.2. INTRODUCTION

Pseudomonas butanovora is a Gram negative, rod-shaped bacterium. It was isolated from activated sludge from an oil refining plant using *n*-butane as the energy source (Takahashi *et al.*, 1980). Based on morphological, physiological and biochemical characteristics, this organism was grouped in the genus *Pseudomonas* (Takahashi, 1980). However, the sequence of the 16S ribosomal DNA gene is most similar to that of members of the genus *Azoarcus*¹. This genus is characterized by members that degrade aromatic compounds (Krieger *et al.*, 1999; Philipp & Schink, 1998) under anaerobic conditions and other members that are plant epiphytes with the ability to fix nitrogen (Reinhold-Hurek *et al.*, 1993). *P. butanovora* can utilize a variety of organic compounds as the growth substrate: C₂-C₉ *n*-alkanes, the corresponding primary alcohols, carboxylic acids and some polyvalent alcohols, but not alkenes, sugars or C₁ compounds (Takahashi, 1980; Takahashi *et al.*, 1980). Butane-grown *P. butanovora* can degrade several chlorinated aliphatic hydrocarbons (Hamamura *et al.*, 1997), which indicates the potential of butane-grown *P. butanovora* in the bioremediation of sites contaminated with solvents.

Except for the gram-negative bacteria, *P. butanovora* and *Pseudomonas* sp. strain CRL71 (Hou *et al.*, 1983), most butane-oxidizing bacteria are members of the gram positive *Corynebacterium-Nocardia-Mycobacterium-Rhodococcus* complex (Ashraf *et al.*, 1994). The pathways for metabolism of gaseous *n*-alkanes (C₂-C₄) by some of these organisms have been investigated by several techniques. In all cases, metabolism is initiated by a monooxygenase (Ashraf *et al.*, 1994; Hamamura *et al.*, 1999; Perry, 1980; Stephens & Dalton, 1986; Woods & Murrell, 1989). Terminal oxidation of an alkane leads to the corresponding 1-alcohol while

¹ N. Hamamura, personal communication

subterminal oxidation produces the 2-alcohol. Evidence for either, and in certain organisms for both, terminal and subterminal oxidation has accumulated. For example, the terminal oxidation of butane was inferred by the induction of isocitrate lyase in *Mycobacterium vaccae* JOB5 grown on butane or butyrate. Furthermore, isocitrate lyase was not induced when cells were grown on butanone, a predicted intermediate in the subterminal oxidation of butane (Phillips & Perry, 1974). In contrast, subterminal oxidation of propane was suggested by the accumulation of acetone in propane-grown cells (Vestel & Perry, 1969). Moreover, an inducible 2-propanol dehydrogenase was purified from *M. vaccae* JOB5 grown on either propane or 2-propanol (Coleman & Perry, 1985). *Pseudomonas fluorescens* NRRL-B1244 can utilize propane, 1-propanol and 2-propanol as growth substrates. Two soluble NAD⁺-linked alcohol dehydrogenases (ADHs) (one primary ADH and one secondary ADH) were detected in propane-grown cells, which suggested that propane is metabolized through both terminal and subterminal oxidation pathways (Ashraf & Murrell, 1992). The presence of both terminal and subterminal propane oxidation pathways in *Rhodococcus rhodochrous* PNKb1 was suggested because mutants unable to utilize either 1-propanol or 2-propanol were also unable to use propane as a growth substrate, indicating that both alcohols are intermediates of propane metabolism (Ashraf & Murrell, 1992). The terminal oxidation of butane by butane-grown *Nocardia* TB1 was indicated by the accumulation of butyrate in the presence of appropriate inhibitors (Van Ginkel *et al.*, 1987). These results indicate the diversity of butane and propane oxidation pathways including the diversity of the alcohol dehydrogenases involved in alkane metabolism.

The pathway of butane metabolism by butane-grown *P. butanovora* was recently determined to follow the terminal oxidation pathway, that is butane → 1-butanol → butyraldehyde → butyrate (Arp, 1999). Each intermediate 1) accumulated in the presence of appropriate inhibitors, 2) supported cell growth,

and 3) stimulated O₂ consumption of butane-grown cells. Although no production of 2-butanol was detected, 2-butanol was consumed and stimulated O₂ consumption by butane-grown *P. butanovora*. Beers (1988) examined the expression of ADH in butane-grown *P. butanovora*. Three soluble ADHs (one primary ADH and two secondary ADHs) that required NAD⁺ as an electron acceptor were found. A membrane-bound, NAD(P)⁺-independent ADH was also described.

In this study, we focused on the characterization of the ADH(s) induced in *P. butanovora* in response to growth on butane. Although butane-grown cells can consume 1- and 2-butanol, it was not known if one ADH was oxidizing both substrates. Our goal was to determine the number and specificity of ADHs present in butane-grown *P. butanovora* and to purify and characterize the inducible ADH primarily involved in the butane metabolism pathway. We report here on the purification and characterization of a quinoxemoprotein, type II ADH induced in butane-grown *P. butanovora*. This report is the first to describe a quinoxemoprotein produced from *n*-alkane-grown bacteria.

2.3. MATERIALS AND METHODS

2.3.1. Chemicals

All high purity alcohols and aldehydes (98%-99.99%) were purchased from Sigma and Aldrich. Other chemicals used were analytical grade.

2.3.2. Bacterial strains and growth condition

Pseudomonas butanovora (ATCC 43655) cultures were grown in 12L bottles at 30 °C with vigorous recirculation of a gas mixture (150 L consisting of 10% (v/v) butane, 5% (v/v) CO₂, 85% (v/v) air) through the bottle. The growth medium consisted of, per liter, 8.0 g (NH₄)₂HPO₄, 1.9 g Na₂HPO₄·7H₂O, 2.0 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 0.06 g CaCl₂·2H₂O, 0.05 g yeast extract, and 1 ml trace element described previously (Wiegant & de Bont, 1980). For growth on 1-butanol, 2-butanol, or lactate, an appropriate amount of each growth substrate was added into the medium to make the final concentration to 4 mM. Butane and CO₂ were omitted from the gas mixture. Cells were harvested at mid to late exponential growth phase (~45-48 hours). Cells were harvested by centrifugation (JA-14, Beckman J2-21), (15 min at 11,000 g, 4 °C), resuspended 1:1 (w/v) with 25 mM Tris-HCl, pH 8.0 and kept at -80 °C until used.

2.3.3. Preparation of soluble fraction and purification of 1-butanol dehydrogenase (BDH)

All procedures for preparation of the soluble fraction and further purification were performed at 4 °C unless stated otherwise. Unbroken cell suspensions were passed three times through a French pressure cell at 55 MPa to 62 MPa. Unbroken cells were removed by centrifugation at 11,000 g, 15 min. The cell-free extract was centrifuged at 200,000 g for 1 hour (SW40, Beckman L8-70), and the resulting supernatant was the soluble fraction and the sediment was collected as the membrane fraction.

BDH was purified from the soluble fraction prepared from butane-grown cells (see above) by the following steps. In each step, the active fraction(s) were

selected primarily by activity stain of non-denaturing gels and then the 1-butanol-dependent PMS reductase activity of each active fraction was quantified spectrophotometrically. All active fractions were pooled and dialyzed against 25 mM Tris-HCl buffer (pH 8.0) at 4 °C overnight before being applied to the next column. (i) Fractionation with ammonium sulfate: To the soluble fraction, ammonium sulfate (to 30% saturation) was gradually added. The resulting precipitate was removed by centrifugation at 14,000 *g*, 30 min. Additional ammonium sulfate was added to the supernatant to reach 70% saturation. The resulting precipitate was collected by centrifugation and re-dissolved in two fold (v/w) of 25 mM Tris-HCl buffer (pH 8.0). (ii) a Q-Sepharose FPLC: The dialyzed 30-70% ammonium sulfate fraction was applied to Q-Sepharose anion exchange FPLC column (Millipore Corp.) (2.2 x10 cm) which had been equilibrated with 25 mM Tris-HCl buffer, pH 8.0. The proteins were eluted by continuous gradient of 0 to 1 M NaCl in the same buffer. The ADH of interest, which has a pinkish-red color, eluted at 200-250 mM NaCl, while a secondary ADH eluted at approximately 600 mM NaCl. (iii) 4-amino-1-butanol affinity chromatography: The dialyzed Q-Sepharose fraction containing a primary ADH was applied to 4-amino-1-butanol substrate-analog affinity column prepared as previously described (Beers, 1988; Lange & Vallee, 1976). The proteins were eluted by a step gradient of 0.05 M, 0.075 M, 0.1 M, 0.2 M, and 1 M NaCl in 25 mM Tris-HCl, pH 8.0. Most proteins did not bind to the column. The ADH of interest eluted at 0.05 M NaCl.

2.3.4. Enzyme assays

All enzyme assays were performed at 25 °C. NAD⁺-independent ADH activity was routinely measured as the phenazine methosulfate (PMS) reductase

activity. PMS reductase activity was measured spectrophotometrically by monitoring the reduction of 2,6-dichlorophenol indophenol (DCPIP) at 600 nm in a reaction mixture consisting of 25 mM Tris-HCl (pH 8.0), 0.7 mM PMS, 0.1 mM DCPIP, 4 mM NH_4Cl , 0.8 mM KCN and 1 mM 1-butanol in a total volume of 3 ml. The enzyme activity of NAD^+ -dependent ADH was measured by the increase in A_{340} due to NADH in a reaction mixture (total volume 3 ml) consisting of 25 mM Tris-HCl (pH 8.0), 2 mM NAD^+ and the enzyme solution. The endogenous rate of DCPIP or NAD^+ reduction obtained without substrate was subtracted from the rate observed with substrate. The reaction mixture (2 ml) for ferricyanide reductase activity assay contained 25 mM Tris-HCl, pH 8.0, 1 mM potassium ferricyanide, 1 mM 1-butanol, and enzyme solution. The enzyme concentration was fixed at 50 nM. One unit of the enzyme activity was defined as the amount of enzyme catalyzing oxidation of 1 μmole of substrate per minute under the conditions described. Protein content was estimated by the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL) with bovine serum albumin as a standard (Smith *et al.*, 1986).

2.3.5. Determination of 1-butanol oxidation and product formation by gas chromatography

The concentration of substrate (alcohol or aldehyde) utilized and type of the products (aldehyde or organic acid) formed were determined by Shimadzu GC-8A gas chromatography equipped with a flame-ionization detector and 60 cm long by 0.1 cm inside-diameter stainless steel column packed with Porapak Q resin. The column temperature was 150 °C for detection of 1-butanol and butyraldehyde, and 120 °C for detection of 2-butanol and butanone.

2.3.6. Analytical gel filtration

The molecular weight of native protein was determined by gel filtration (column 1.6 by 30 cm) with Sephacryl S-300 (Pharmacia). Elution was performed at a flow rate 0.5 ml min^{-1} with 25 mM Tris-HCl buffer, pH 8.0 containing 0.15 mM NaCl and monitored at A_{280} . The following molecular weight marker proteins were used for calibration: catalase (M_r 240,000), bovine serum albumin (M_r 66,000), carbonic anhydrase (M_r 30,000), and cytochrome c (M_r 13,000).

2.3.7. Electrophoresis

10% SDS-PAGE (Mighty Small™ SE245, Hoefer Scientific Instruments) (Laemmli, 1970) and Coomassie brilliant blue G250 staining were used to analyze the homogeneity of the purified enzyme.

2.3.8. Non-denaturing gel and activity staining

Native protein was applied to non-denaturing gels prepared as above but without SDS. After PAGE, the gel was incubated for 5 min in the dark with a reaction mixture (50 ml) containing 0.01 g nitroblue tetrazolium (NBT), together with 0.7 mM PMS or 1 mM NAD^+ alone or in combination. Then, either 1-butanol or 2-butanol was added to a final concentration of 1 mM and the gel was incubated with gentle rocking for another 5 min to develop the color. The reaction was stopped by rinsing the gel with water.

2.3.9. Kinetic measurements

The kinetic parameters were calculated from the initial rates determined with the PMS reductase standard assay described above by varying the concentrations of substrates tested from 2 μ M-1 mM. The enzyme concentration was fixed at 50 nM.

2.3.10. Preparation of antibodies and immunoblotting

The purified BDH (2 x 100 μ g) well mixed 1:1 (v/v) with TiterMax adjuvant (Sigma) was injected into two New Zealand rabbits. Two weeks later, a boost injection of another 100 μ g of protein mixed with TiterMax was performed. Another two weeks later, blood was collected, then left at room temperature for 30 min and overnight at 4 °C. After centrifugation at 1,000 g, 10 min to remove the erythrocytes, the antisera were obtained and stored at -80 °C.

For immunoblotting analysis, the proteins in the SDS gel after PAGE were transferred electrophoretically with a semidry system (Ausubel *et al.*, 1996) onto a polyvinylidene difluoride (PVDF) membrane at 10 mA overnight. The membrane was washed with 15 ml Tris-buffered-saline (TBS) (20 mM Tris-HCl, 150 mM NaCl pH 7.5) before blocking with 15 ml of 1% BSA in TBST (TBS with 0.1% Tween 20) for 1 hr. Then, the membrane was incubated with primary antiserum (dilution 1:5,000 in TBST) for 1 hr. The membrane was washed twice with TBST before incubation with TBST containing secondary antibody (1 mg Anti-rabbit IgG (FC) ml⁻¹, Promega #4265805) conjugated with alkaline phosphatase (dilution 1:5,000 in TBST) for 30 min. After the membrane was washed with 20 ml TBST three times, the immunosorbent protein bands were visualized by the addition of

15 ml of Western blue[®] solution (Promega). The color was developed within 5-20 min. Pre-stained molecular weight marker proteins (Bio-Rad) were included.

2.3.11. Heme staining

A lithium dodecylsulfate (LDS)-PAGE gel was prepared and pre-run overnight (Delepelaire & Chua, 1979). Purified BDH was then applied to the gel and run at 5 mA. Heme staining was performed as previously described (Thomas *et al.*, 1976).

2.3.12. Measurement of heme c content

The amount of heme c in the purified BDH was measured from the reduced minus oxidized difference spectra of the pyridine hemochrome (Paul *et al.*, 1953), using a millimolar extinction coefficient of 26.5 ($\Delta\epsilon$ of 550 nm-534 nm). The oxidized form of pyridine hemochrome was prepared by adding pyridine to 20% (v/v), NaOH to the final concentration of 0.2 N, and potassium ferricyanide to the final concentration of 3 mM, to the enzyme solution. To reduce the reaction mixture, 2 mg sodium dithionite was added into the completely oxidized pyridine hemochrome solution. Cytochrome c from horse heart (Boehringer Mannheim) was used to test the protocol and gave a value of 1.0 heme per protein molecule.

2.3.13. Measurement of PQQ content

PQQ content in purified BDH was determined by HPLC analysis as previously described (Duine *et al.*, 1983).

2.3.14. N-terminal and internal amino acid sequence analysis

To separate the upper and lower bands of the purified BDH, a 13 cm by 13 cm SDS gel was used. Both upper and lower bands were then blotted onto PVDF membrane. The N-terminal amino acid sequences were then determined directly. The internal amino acid sequence was obtained by digesting the purified BDH with endoproteinase lys C (Endo Lys-C) in the reaction mixture consisting of 0.2 M Tris pH 9.1, 20% acetonitrile. The enzyme was digested at 37 °C for 20 hr, then DTT was added to the final concentration of 5 mM and incubated at 37 °C for another 20 hr. Peptides in the digest were separated by HPLC using a Vydac C18 column with a Brownlee guard column. A mobile phase A was 0.1% trifluoroacetic acid (TFA) and a mobile phase B was 70% acetonitrile and 0.1% TFA. Chromatography was performed with a gradient from 1%B to 65%B in 75 min. Peaks were monitored at 220 nm. and collected for sequence analysis. The prominent fragment was selected for sequencing. The N-terminal and internal amino acid sequencing including enzyme digestion were determined by the Biotechnology Lab, Institute of Molecular Biology, University of Oregon.

2.4. RESULTS

2.4.1. Multiple ADHs in *Pseudomonas butanovora*

Cell supernatants from cells grown on different substrates were examined for ADH activities using 1-butanol or 2-butanol as substrate. Four distinct activity bands were identified on gels stained for ADH activity when 2-butanol was used as a substrate (Fig. 2.1G). ADH#1, which is the enzyme activity band with the fastest mobility, was detected in the presence of PMS when either 1-butanol (Fig. 2.1C) or 2-butanol (Fig. 2.1F) was added. This activity band was not present with either 1- or 2-butanol when NAD^+ was used without PMS (Fig. 2.1B, E) and the presence of NAD^+ together with PMS did not enhance the band intensities (Fig. 2.1D, G). Therefore, the activity of ADH#1 is NAD^+ -independent. The intensity of this band varied with the source of the cells. The intensity of ADH#1 activity was highest in butane-grown cells and lowest in lactate-grown cells (Fig 2.1C, D; lane 3 and 4, respectively). The activity of ADH#1 was much less when 2-butanol rather than 1-butanol was used as the substrate (Fig. 2.1F, G). Therefore, this enzyme appears to be a primary ADH and has been designated as 1-butanol dehydrogenase (BDH). Three ADHs (ADH#2, #3, and #4) were identified as secondary ADHs since they were detected only when 2-butanol was used as a substrate (Fig. 2.1G). None of these secondary ADHs were detected in 1-butanol-grown cells while one to two secondary ADHs were detected in 2-butanol-grown, butane-grown and lactate-grown cells (Fig. 2.1G). All three cell extracts contain ADH#4, which has the slowest mobility in the non-denaturing gel. 2-Butanol-grown cells and lactate-grown cells contained additional secondary ADHs, indicated as ADH#2 and ADH#3, respectively. Butane-grown cells exhibited little ADH#2 activity and no ADH#3 activity. These secondary ADHs (ADH#2, #3, and #4) all required NAD^+

for activity and are therefore considered NAD^+ -dependent. Although no activity was observed with PMS alone, the NAD^+ -dependent activity also required the presence of PMS (Fig. 2.1E, F, G). The failure to observe activity with NAD^+ alone most likely indicated that NAD^+ cannot act as sole electron acceptor under condition tested and indicated a role for PMS in mediating electron transfer from NADH to nitroblue tetrazolium (Hisada & Yagi, 1977; Nakamura *et al.*, 1982). The slowly migrating bands that appeared in both the presence and absence of butanol were not attributed to butanol dehydrogenase activity and likely came from nonspecific interactions, known as nothing dehydrogenase, of the nitroblue tetrazolium with proteins on the gels (Van Noorden *et al.*, 1985).

2.4.2. 1-Butanol dehydrogenase is induced during growth of *P. butanovora* on butane

Growth substrate-dependent differences in the induction of primary and secondary alcohol dehydrogenase activities in *P. butanovora* were apparent from the activity-stained non-denaturing gels (Fig. 2.1C, D). To quantify these differences, BDH activity was determined with the spectrophotometric assay in extracts of cells grown on butane, 1-butanol, 2-butanol or lactate. The highest activity was present in cells grown on butane ($32.9 \pm 1.7 \text{ nmole} \cdot \text{min}^{-1} \cdot (\text{mg protein})^{-1}$). The activities of extracts of cells grown on 1-butanol, 2-butanol or lactate were 10.6 ± 0.5 , 4.4 ± 0.1 and $1.9 \pm 0.1 \text{ nmole} \cdot \text{min}^{-1} \cdot (\text{mg protein})^{-1}$, respectively. The activities determined in solution correlated with the intensities of BDH activity band on native gels.

To further confirm the correlation of BDH activity with butane metabolism, the induction of BDH upon exposure of cells to butane was examined. Cells were grown in citrate (5 mM) because this substrate does not repress the induction of butane metabolism when butane is provided. In contrast, lactate and succinate

induce catabolic repression in *Pseudomonas* (Duetz *et al.*, 1994; Yuste *et al.*, 1998). When cells reached an OD₆₀₀ of 0.6 (2 days), they were divided into 3 parts. One part remained in the citrate-containing medium, another part received 10% butane in the gas phase, and the third part was supplemented with 1-butanol (to 4 mM). Cells were incubated at 30 °C with shaking. After 6 hours and 12 hours, samples of cells were harvested, washed, and broken. Further investigation of the induction of BDH activity was performed by Western blot analysis. The results from the activity assay are consistent with the increases of cross-reacting band intensities in Western blot analysis. As shown in Fig. 2.2A, the cross-reacting band of an inducible BDH was first seen after cells were incubated with 1-butanol for 6 hours. After 12 hr clearly visible cross-reacting bands were present in cell-free extracts of 1-butanol-induced and butane-induced cells, but not in citrate-grown cells. The 1-butanol-PMS/DCPIP-dependent activity assay (Fig. 2.3B) showed 22- fold and 30-fold increases of BDH activity after citrate-grown cells were induced by 1-butanol and butane for 12 hr, respectively.

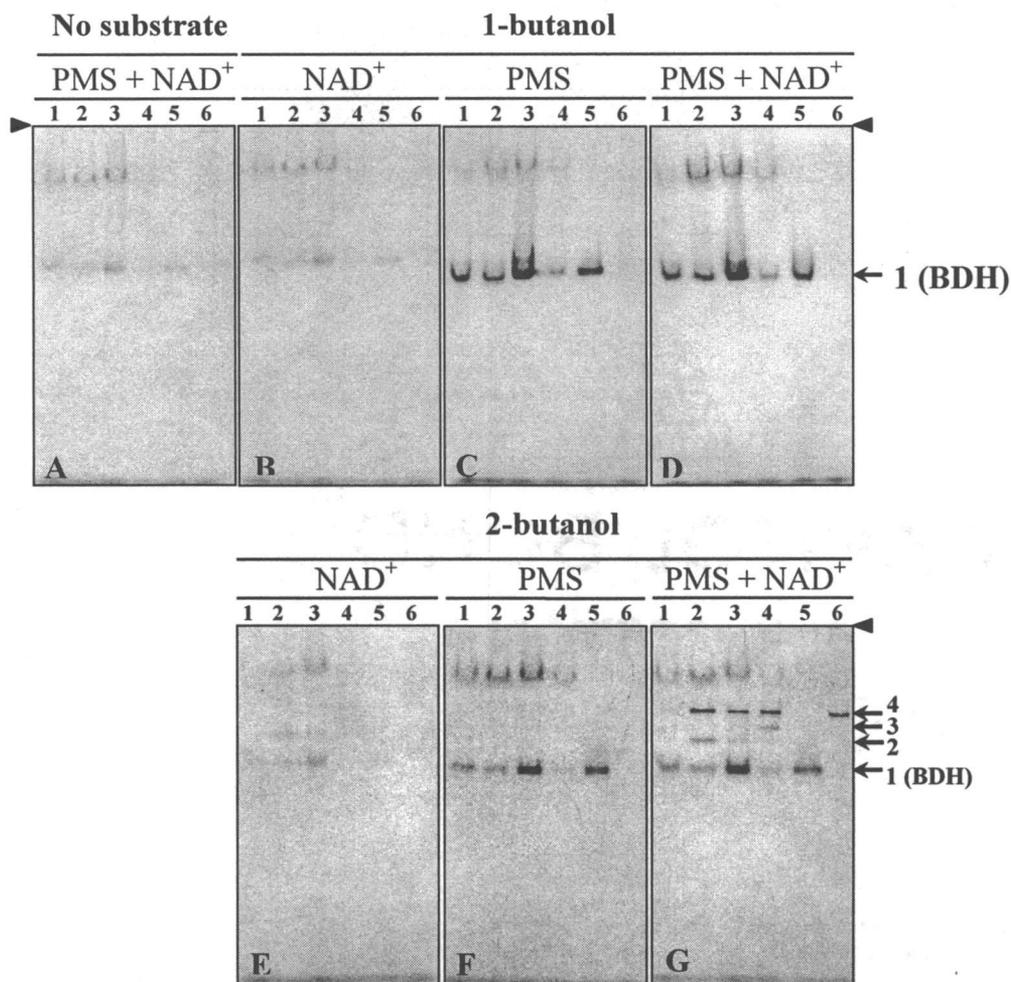


Figure 2.1 Non-denaturing gels stained for alcohol dehydrogenase activity. Native gels after PAGE were stained for 5 min with nitroblue tetrazolium (NBT) with: NAD⁺ alone (B,E); PMS alone (C, F); PMS and NAD⁺ together (A, D, G). Then, gels were incubated for additional 5 min with: No substrate (A); 1 mM 1-butanol, (B, C, D); 1 mM 2-butanol, (E, F, G). Lanes 1-4 contain cell-free extracts from cells grown in: 1-butanol (18 μ g protein), lane 1; 2-butanol (22 μ g protein), lane 2; butane (19 μ g protein), lane 3; and lactate (22 μ g protein), lane 4. Lane 5 contained the purified 1-butanol dehydrogenase (BDH) (0.5 μ g protein), and lane 6 contained partially purified secondary alcohol dehydrogenase (20 μ g protein) from *P. butanovora*. The interface of a stacking gel and a resolving gel are indicated by triangles. Bands corresponding to four ADHs found in cell-free extracts are indicated.

2.4.3. BDH activity is in the soluble fraction

Our results suggested that an NAD^+ -independent primary ADH (i.e. BDH) was primarily responsible for 1-butanol oxidation. In contrast, it was previously reported that the enzyme responsible for 1-butanol oxidation in butane-grown *P. butanovora* was a membrane-bound NAD(P)^+ -independent primary ADH (Beers, 1988). Therefore, we carefully examined the membrane fraction for ADH activity. Cells grown on 10% butane were harvested, broken, then soluble and membrane fractions were separated as described in Materials and Methods. Membrane fractions from different cell growth-stages (the beginning of exponential phase, OD_{600} 0.3-0.5; mid-late exponential phase, OD_{600} 0.6-0.8; and stationary phase, OD_{600} 1.5-1.7) and solubilized in various concentrations (0.05%, 0.1%, 0.5% and 1%) of each of several detergents (Triton X-100, Emulgen and dodecyl- β -D-maltoside) were tested. None of these preparations showed significant BDH activity (less than 2.5% of the specific activity obtained from soluble fraction) whether NAD^+ or PMS/DCPIP was used in the activity assay (data not shown). Therefore, the soluble fraction from butane-grown *P. butanovora* was used as an enzyme source for BDH purification.

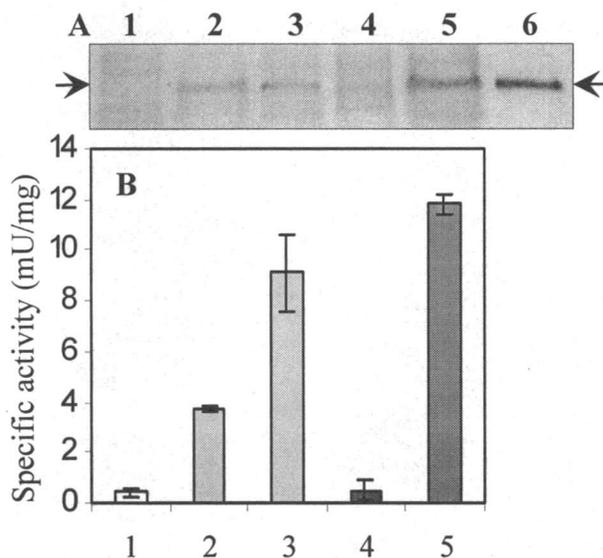


Figure 2.2 Induction of BDH during growth of *P. butanovora* on 1-butanol and butane. **(A)** Western-blot analysis of SDS-PAGE of cell-free extracts of *P. butanovora* grown in 5 mM citrate and induced for the BDH activity by 1-butanol or butane for 6 and 12 hr. The antibodies used were raised against purified 1-butanol dehydrogenase from butane-grown *P. butanovora*. Lane 1-5 contain cell-free extracts from: 12 hr citrate-grown cells (22 μ g protein) (lane 1), 6 hr 1-butanol-induced cells (20 μ g protein) (lane 2), 12 hr 1-butanol-induced cells (20 μ g protein) (lane 3), 6 hr butane-induced cells (18 μ g protein) (lane 4), and 12 hr butane-induced cells (23 μ g protein) (lane 5). Lane 6 contains purified 1-butanol dehydrogenase (2 μ g protein). **(B)** 1-butanol-PMS/DCPIP-dependent activity assay of cell-free extracts from: 12 hr citrate-grown cells (1), 6 hr 1-butanol-induced cells (2), 12 hr 1-butanol-induced cells (3), 6 hr butane-induced cells (4) and 12 hr butane-induced cells (5).

2.4.4. Purification and physical properties of 1-butanol dehydrogenase (BDH)

1-Butanol dehydrogenase activity was the predominant ADH activity in butane-grown cells. The activity was closely correlated with butane metabolism. Therefore, we focused on this enzyme for additional characterization. BDH from butane-grown *P. butanovora* was purified from the soluble fraction to near homogeneity by a six-step procedure (Table 2.1). Many contaminating proteins were removed by the first anion exchange (Q-Sepharose) column followed by butanol affinity column. Repeating these two column fractionations was sufficient to purify the BDH to near homogeneity. The 37-fold increase in specific activity indicated that BDH accounted for about 2.7% of the soluble proteins.

A doublet was observed when the purified enzyme was analyzed by 10%-SDS-PAGE (Fig. 2.3A). The purified BDH was rerun in a large SDS gel (Fig. 2.3B) to separate these two bands (67,000 Da and 65,700 Da) which were then blotted onto PVDF membrane and sequenced. The N-terminal amino acid sequence obtained from each band of BDH revealed that, in fact, they belong to the same enzyme (Table 2.2). This N-terminal amino acid sequence revealed no strong similarity to any N-terminal amino acid sequence in the data banks. This result was not surprising since it has been previously reported that there is generally a low similarity at the N-terminal part of this type of proteins (Stoorvogel *et al.*, 1996; Zarnit *et al.*, 1997). To obtain internal amino acid sequences from BDH, an endoproteinase lys C digestion was performed and two fragments were purified by HPLC and sequenced. Two alignments of the internal amino acid sequences showed high similarities to parts of quinohemoprotein ethanol dehydrogenase (EDH) from ethanol-grown *C. testosteroni*: one having an identity of 73% and a similarity of 83% to a part of EDH amino acid sequence (residues 427-446) and the other one having an identity of 62% and a similarity of 66% to a part of the EDH amino acid sequence (residues 646-670) (Table 2.2). The secondary ADH observed

in butane-grown cells (ADH#4) was also partially purified from the soluble fraction through the step of anion exchange chromatography (Fig. 2.1, lane 6). The partially purified ADH#4 did not oxidize 1-butanol (either with NAD^+ or PMS), but oxidized 2-butanol when NAD^+ was provided.

The molecular mass of BDH, the primary alcohol dehydrogenase, was estimated to be 66 kDa by SDS-PAGE (Fig. 2.3) and 69 kDa by gel permeation chromatography (Sephacryl S-300), which indicates that BDH exists as a monomer. This characteristic is similar to other monomeric quinohemoproteins with a range of molecular mass of 69-73 kDa (Groen *et al.*, 1986; Hopper *et al.*, 1991; Toyama *et al.*, 1995; Zarnt *et al.*, 1997). Because of the sequence similarity to the quinohemoprotein, ethanol dehydrogenase; the pyrrolo-quinoline quinone (PQQ) content of BDH was examined. BDH contains almost 1:1 molar ratio of PQQ (0.99 nmole per nmole protein) which indicates that PQQ was not lost during the purification. It has been reported that PQQ is non-covalently bound to quinohemoprotein-type enzymes and can be dissociated by dialysis overnight in Tris-HCl buffer, pH 8.0 containing 2 mM EDTA (Zarnt *et al.*, 1997) or by heat treatment (Toyama *et al.*, 1995).

BDH exhibits a pinkish-red color in solution and appears as a red band in non-denaturing gels. A heme-staining band corresponding to a molecular mass of 65 kDa (data not shown) suggested the presence of heme cofactor in this enzyme. BDH as isolated exhibited a typical spectrum of reduced c-type cytochrome with absorption peaks at 416, 520 (β -peak) and 550 (α -peak) nm (Fig. 2.4). When BDH was oxidized with 200 μM potassium ferricyanide, the α and β peaks (550 and 520 nm) disappeared and the γ -peak (416 nm) was shifted to 410 nm (Fig. 2.4). This spectral change from the reduced form to oxidized form is characteristic of cytochrome c.

Table 2.1 Purification steps and specific activity of 1-butanol dehydrogenase from butane-grown *P. butanovora*.

Purification steps	Total protein (mg)	Total activity* (U) [†]	Specific activity (U.mg ⁻¹) [†]	Purification (-fold)	Recovery (%)
Crude supernatant	1921	250	0.13	1	100
30-70% Ammonium sulfate precipitation	840	308	0.37 ± 0.01	3	123
First Q-Sepharose column fractionation	134	208	1.6 ± 0.06	12	83
First 4-amino-butanol affinity column fractionation	43	143	3.3 ± 0.02	26	57
Second Q-Sepharose column fractionation	16	55	3.5 ± 0.04	27	22
Second 4-amino-butanol affinity column fractionation	7	33	4.8 ± 0.34	37	13

* Activity was determined as 1-butanol dependent PMS/DCPIP reduction.

[†] One unit (U) of activity was defined as the reduction of 1 μmole of DCPIP per min under the specific conditions of the assay described in Materials and Methods.

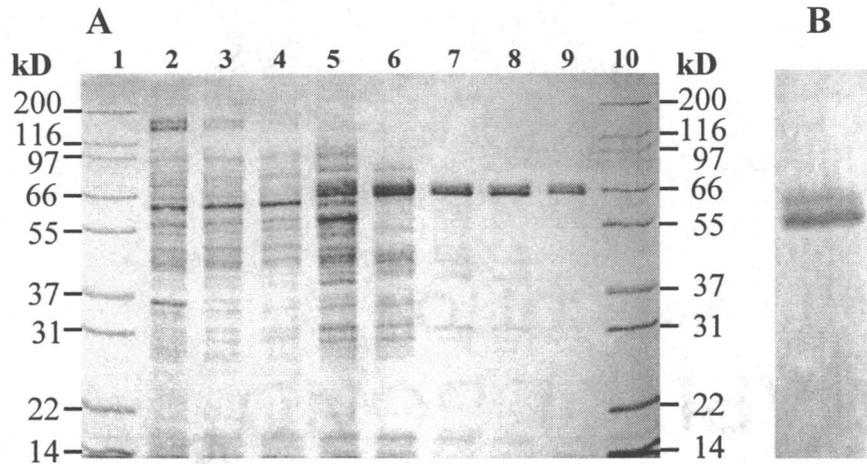


Figure 2.3 10%-SDS-PAGE and Coomassie blue stain of BDH from butane-grown *P. butanovora* after purification steps. All lanes contained approximately 5 μ g protein. (A) Lane: 1 and 10 molecular weight markers; 2 cell-free extract; 3 crude supernatant (after membrane fraction was removed); 4 30%-70% ammonium sulfate precipitation; 5 after first Q-Sepharose column fractionation; 6 after first 4-amino-butanol affinity column fractionation; 7 after second Q-Sepharose column fractionation; 8 after second 4-amino-butanol affinity column fractionation; 9 Sephacryl S-300 (for molecular weight verification and subunit determination purposes). (B) The purified BDH was re-run in a large SDS gel to separate two bands (67,000 Da and 65,700 Da) which were then blotted onto PVDF membrane and sequenced.

Table 2.2 Alignment of the N-terminal and internal amino acid sequences of BDH from butane-grown *P. butanovora* and the quinohemoprotein EDH from *C. testosteroni*

Organism		Amino acid sequences*	Identity (%)	Similarity (%)
<i>P. butanovora</i>	N-terminal upper band	AGGEWRT-GYD-A-T		
	N-terminal lower band	AGGEWRTAGY-DA-T		
<i>P. butanovora</i>	Internal sequence	... K PMSYAP Q TGLAY F PA Q N I P...	73	83
<i>C. testosteroni</i>	Internal sequence (427-446)	...HPMSFNP Q TGLVY L PA Q N V P...		
<i>P. butanovora</i>	Internal sequence	... K GG G IP N LG Y ST A ET I A H LD Q F V F K ...	62	66
<i>C. testosteroni</i>	Internal sequence (646-670)	... R GG N IP N LG Y MD A S Y I E N L PN F V F K...		

*Bold letters indicates identical residues

The BDH absorption spectrum resembles the absorption spectra obtained in other quinohemo-proteins, for example, the absorption spectra of lupanine hydroxylase with peaks at 416, 522, 551 nm (Hopper *et al.*, 1991); ADH of *P. putida* HK5 with peaks at 418, 523, 550 nm (Toyama *et al.*, 1995); Tetrahydrofurfuryl ADH from *R. eutropha* with peaks at 419, 523, 552 (Zarnt *et al.*, 1997) and that of holoenzyme EDH from *C. testosteroni* (De Jong *et al.*, 1995). When 1 mM 1-butanol was added to the oxidized enzyme, it acted as an electron donor and reduced the c-type heme in BDH (Fig. 2.4). This result indicated the participation of a cytochrome c-type prosthetic group in the catalytic activity of BDH. From the "reduced minus oxidized" difference spectrum of pyridine hemochrome (at the absorbance at 534 nm and 550 nm), a ratio of heme per enzyme molecule was calculated. One mole heme per 3.8 mole BDH enzyme was obtained. Typically, quinohemoproteins contain heme with 1:1 molar ratio (de Jong *et al.*, 1995; Toyama *et al.*, 1995; Zarnt *et al.*, 1997). Although we cannot exclude the possibility, a loss of heme during purification seems unlikely given that c-type hemes are generally covalently attached to the protein. Perhaps the substoichiometric heme c content reflects an incomplete assembly of heme c into BDH under the conditions used for growing cells. The presence of two forms of BDH, one with and one without heme c, likely explains the presence of two bands on SDS-PAGE gels (Fig. 2.3A, 2.3B).

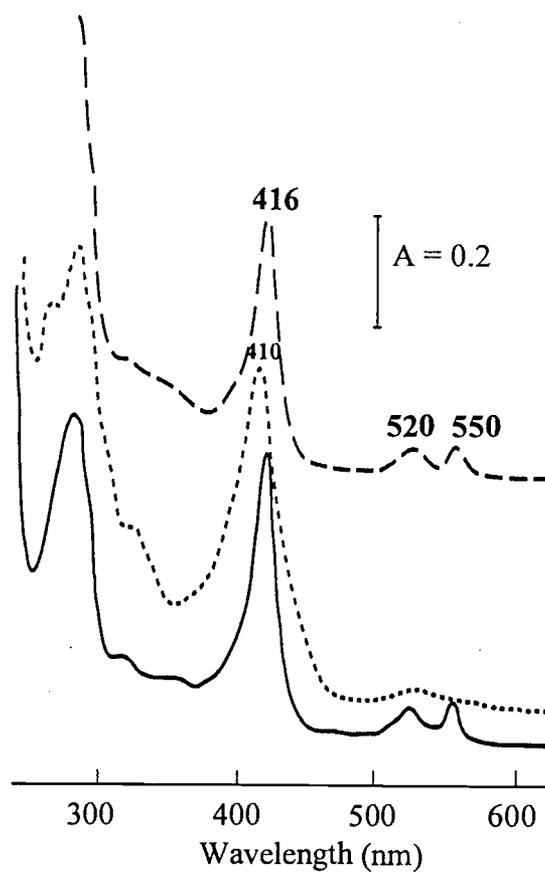


Figure 2.4 UV/visible spectra of purified 1-butanol dehydrogenase (BDH) (10 μM) from butane-grown *P. butanovora* measured in 25 mM Tris/HCl, pH 8.0. —, Purified BDH; ---, BDH oxidized with 200 μM potassium ferricyanide; -·-, BDH re-reduced by the addition of 1 mM 1-butanol to the oxidized enzyme.

2.4.5. Catalytic properties of BDH

In addition to 1-butanol, PMS/DCPIP activity, we also examined BDH for its ferricyanide reductase activity, which is one of the characteristics of quinohemoproteins. The ferricyanide reductase activity exhibited by BDH from butane-grown *P. butanovora* was $0.62 \pm 0.2 \mu\text{mole} \cdot \text{min}^{-1} \cdot (\text{mg protein})^{-1}$ when 1-butanol was used as a substrate.

The effect of calcium ion on BDH catalytic activity was determined. The presence of Ca^{2+} ion facilitated the reconstitution of inactive apoenzymes (PQQ-free, but heme *c*-containing) quinohemoprotein from *C. testosteroni* and alkaloid-degrading *Pseudomonas* and increased the ADH catalytic activity (De Jong *et al.*, 1995; Groen *et al.*, 1986; Zarnt *et al.*, 1997). Ca^{2+} fulfills a structural role upon reconstitution of apoenzyme with PQQ, facilitating the binding process of PQQ to an apoenzyme and activates the bound cofactor (Groen *et al.*, 1986; Zarnt *et al.*, 1997). The activity of BDH from butane-grown *P. butanovora* was significantly increased when Ca^{2+} was added into the reaction mixture. BDH activity with 1-butanol was increased from $3.4 \pm 0.2 \mu\text{mole} \cdot \text{min}^{-1} \cdot (\text{mg protein})^{-1}$ (without Ca^{2+}) to $4.2 \pm 0.01 \mu\text{mole} \cdot \text{min}^{-1} \cdot (\text{mg protein})^{-1}$ with 5 mM CaCl_2 and $4.6 \pm 0.2 \mu\text{mole} \cdot \text{min}^{-1} \cdot (\text{mg protein})^{-1}$ with 10 mM CaCl_2 . The maximal and saturated activity was reached at the calcium ion concentration of 10 mM. As recently reported, Ca^{2+} was found to have an important role in the catalytic mechanism of quinoprotein-type methanol dehydrogenase (Zheng & Bruice, 1997). The possible roles of Ca^{2+} complexed with PQQ in methanol oxidation consist of: (i) modest reduction of the pK_a of the substrate and facilitating the association of substrate to active site, (ii) polarizing the oxygen at the C-5 position of PQQ, and (iii) placing the reaction components in the right positions to react, therefore contributing to the formation of enzyme-substrate complex (Zheng & Bruice, 1997).

BDH activity was increased approximately 3 fold when ammonium ions (as 4 mM NH_4Cl) were present. A similar result was obtained in tetrahydrofurfuryl ADH from *R. eutropha* where the activity was increased 2.5 fold in the presence of ammonium sulfate (Zarnt *et al.*, 1997).

The optimum pH for PMS-DCPIP-dependent BDH activity was pH 8.0. The temperature optimum was 60 °C at which the specific activity was increased 9.6-fold relative to 25 °C. The temperature range of maximum stability of the enzyme was 25-32 °C where the loss of BDH activity was insignificant after a 30-min incubation. In contrast, BDH activity was decreased 77% when incubated at 60 °C for the same period of time. BDH activity was stable for more than 6 months when stored in 25 mM Tris-HCl, pH 8.0 at -80 °C.

BDH exhibited broad substrate specificity towards various primary alcohols, secondary alcohols, diols, aldehydes and aromatic alcohols (Table 3). Nonetheless, the highest activity was observed with 1-butanol, the butane oxidation product in the terminal oxidation pathway. Therefore, the activities examined from other substrates were compared to the maximal activity obtained from 1-butanol. Besides 1-butanol, 1-propanol showed a relatively high activity (66%) among the primary alcohols tested. The relative activities among primary alcohols decreased with longer chain alcohols. Little activity (1%-2%) was detected with methanol, which is consistent with the observation that *P. butanovora* cannot grow with either methane or methanol as a substrate (Takahashi, 1980). Among the secondary alcohols tested, BDH exhibited a marked preference towards 2-pentanol and the activity gradually decreased with longer chain secondary alcohols. DCPIP was slowly reduced by BDH when 2-propanol or 2-butanol was added. In activity-stained native gels, BDH activity bands were also detected when 2-butanol was added (Fig. 2.1F, G). However, when 2-butanol consumption was determined directly by gas chromatography, no 2-butanol consumption was observed.

Table 2.3 Substrate specificity of a purified 1-butanol dehydrogenase from butane-grown *P. butanovora*

Substrate	Relative activity* (%)	Substrate	Relative activity* (%)
Primary alcohols		Diols	
Methanol	2	1,2-Propanediol	4
Ethanol	34	1,3-Propanediol	5
1-Propanol	66	1,2-Butanediol	30
1-Butanol	100	1,3-Butanediol	33
1-Pentanol	45	1,4-Butanediol	24
1-Hexanol	29	2,3-Butanediol	2
1-Heptanol	31	1,4-Pentanediol	1
1-Octanol	19	2,4-Pentanediol	2
1-Nonanol	9		
Secondary alcohols		Aromatic alcohols	
2-Propanol	5	Benzyl alcohol	11
2-Butanol	9	Phenol	13
2-Pentanol	68		
2-Hexanol	56	Aldehydes	
2-Heptanol	49	Acetaldehyde	54
2-Octanol	42	Propionaldehyde	61
2-Nonanol	35	Butyraldehyde	78

* Enzyme activity was determined by PMS/DCPIP reduction as described in Methods. Activities are mean of at least three replicates. Data are expressed relative to a value of 100% for BDH activity with 1-butanol ($4.8 \pm 0.34 \mu\text{mole} \cdot \text{min}^{-1} \cdot (\text{mg protein})^{-1}$).

Furthermore, no butanone formation was detected within the 60-min assay (data not shown). The low level of DCPIP reduction activity obtained upon addition of either 2-propanol or 2-butanol was probably due to the presence of contaminants that supported activity. BDH was active towards C₄ diols when one of the hydroxyl groups was at α -position. Benzyl alcohol and phenol were chosen as the

representative aromatic alcohols and were slowly oxidized by BDH. BDH readily oxidized the three aldehydes we tested, with butyraldehyde showing the highest activity. In fact, only 1-butanol was oxidized more rapidly than butyraldehyde. The kinetic parameters of BDH with 1-butanol and butyraldehyde were determined. At higher concentrations of 1-butanol (>10 mM), the enzyme activity was decreased. 1-Butanol is a better substrate for BDH (K_m 7 ± 1 μ M) than butyraldehyde BDH (K_m 535 ± 13 μ M). The term k_{cat}/K_m is used to compare the efficiency of catalysis of BDH towards different substrates. 1-Butanol was clearly the preferred substrate with a k_{cat}/K_m of 7.2×10^5 $M^{-1}sec^{-1}$, which is 51 fold higher than that of butyraldehyde. The product of 1-butanol oxidation was butyraldehyde as determined by gas chromatography. A time course experiment confirmed that BDH prefers 1-butanol to butyraldehyde. 1-Butanol (1 mM) and butyraldehyde (1 mM) were added together as substrates for BDH in a reaction mixture containing PMS/DCPIP in 25 mM Tris-HCl pH 8.0. The oxidation of 1-butanol and butyraldehyde and the formation of their products were determined by gas chromatography (Materials and Methods). Only 1-butanol was oxidized during the first 60 min which corresponded with an increase in butyraldehyde (data not shown). After 1-butanol was completely oxidized the oxidation of butyraldehyde was initiated and yielded butyric acid. This result is consistent with the 76 fold lower K_m of BDH towards 1-butanol than towards butyraldehyde.

2.5. DISCUSSION

In this work, four ADHs were detected in *P. butanovora* grown in different growth substrates. The level of expression of each ADH depended on the carbon source used in the medium. *P. butanovora* grown on butane expressed two soluble ADHs, an NAD^+ -independent primary ADH and an NAD^+ -dependent secondary

ADH. This NAD^+ -independent primary ADH has high activity and low K_m towards 1-butanol and was expressed to high level in cells grown on butane or 1-butanol. Therefore, this enzyme (BDH) was considered to be of primary importance in the oxidation of 1-butanol produced from the terminal oxidation of butane. However, our results contradict a report in which a membrane-bound NAD(P)^+ -independent ADH with an unusually high activity towards methanol (>60% relative to 1-butanol) was noted to be responsible for the oxidation of butanol in butane-grown *P. butanovora* (Beers, 1988). In our experiments, no 1-butanol oxidation activity could be detected in the membrane fraction. We purified this soluble, NAD^+ -independent primary ADH from butane-grown *P. butanovora* and showed it to be a quinohemoprotein. As pointed out by Toyama *et al.* (1995), there had been no reports of PQQ-containing ADHs associated with alkane oxidation by bacteria. This work is the first report of a quinohemoprotein induced from alkane-grown bacteria. Interestingly, this inducible BDH from butane-grown *P. butanovora* was found to be different from ADHs induced in other alkane-grown *Pseudomonas* or other short, medium or long chain-alkane oxidizing bacteria. ADHs induced in propane-grown *R. rhodochrous* PNKb1, *M. vaccae* JOB5 and *P. fluorescens* NRRL B-1244 were purified and characterized as NAD^+ -dependent secondary ADH(s) (Ashraf & Murrell, 1990; Ashraf & Murrell, 1992; Coleman & Perry, 1985). Heptane- and octane-grown *P. aeruginosa* strain 473 contain soluble NAD(P)^+ -dependent ADH and at least two NAD(P)^+ -independent quinoprotein (without heme *c*) primary ADHs (Van Der Linden & Huybregtse, 1969).

In addition to methanol dehydrogenase, other quinoprotein ADHs found in oxidative nonmethylotrophic bacteria have been classified into three groups (type I-III) according to their molecular properties, catalytic properties and localization (Matsushita *et al.*, 1994). The molecular structure of type I ADH resembles that of methanol dehydrogenase, but the enzyme has very low affinity for methanol.

Type II ADH is a soluble quinohemoprotein. To date, type II ADHs have been purified from five bacteria. Type II ADHs have been purified as the apoenzyme, lacking PQQ, from two organisms: alkaloid-degrading *Pseudomonas* (Hopper *et al.*, 1991) and *C. testosteroni* (De Jong *et al.*, 1995; Groen *et al.*, 1986), and as the active holoenzyme from the following organisms: *P. putida* HK5 (Toyama *et al.*, 1995), *Rhodopseudomonas acidophila* M402 (Yamanaka & Tsuyuki, 1983), and *Ralstonia eutropha* (Zarnt *et al.*, 1997). Type III ADH is a membrane-bound ADH found in the cytoplasmic membrane of acetic acid bacteria.

The characterization of purified BDH from butane-grown *P. butanovora* indicates that this enzyme belongs to type II NAD⁺-independent ADH or quinohemoprotein (Goodwin & Anthony, 1998). This is also the first report of type II NAD⁺-independent ADH synthesized by an *Azoarcus*-like bacterium. PQQ, a prosthetic group in quinohemoprotein, is expected to participate directly in the oxidation of alcohols and aldehydes (Groen *et al.*, 1986). Apo-EDH, lacking PQQ, but containing heme c has no activity with alcohol or aldehyde substrates. The presence of heme c as a functional prosthetic group in BDH was indicated by the following results: (i) A red band which also stained for ADH activity was observed in non-denaturing gels. (ii) Protein corresponding to a molecular mass of 65 kDa in LDS-PAGE also stained positive for heme. (iii) The absorption spectrum of purified BDH with peaks at 416, 520 and 550 nm revealed a typical reduced cytochrome c spectrum that changed to an oxidized spectrum upon addition of ferricyanide, and back to a reduced spectrum upon addition of 1-butanol. (iv) A ferricyanide reductase activity was exhibited by BDH. Quinoproteins lacking heme c, such as methanol dehydrogenase, EDH from *P. aeruginosa* and *P. putida* and soluble glucose dehydrogenase from *E. coli*, do not react with ferricyanide, suggesting that oxidation of the enzyme by ferricyanide occurs at heme c site (Docker *et al.*, 1986; Geerlof *et al.*, 1994; Groen *et al.*, 1984). For a cell extract from 1-butanol-grown *P. putida* HK5, the ratio of 1-butanol-dependent

PMS/DCPIP-reductase activity to ferricyanide-reductase activity was 2:1 (Toyama *et al.*, 1995). BDH from butane-grown *P. butanovora* as isolated expressed, with 1-butanol present, a specific activity ratio of PMS/DCPIP-reductase activity to ferricyanide-reductase activity of 7.8. This specific activity ratio is consistent with the fact that only one fourth of the BDH as isolated contained heme c.

The physical properties of BDH presented here, including high internal sequence similarity with *C. testosteroni* EDH, indicates that BDH belongs to the group of type II quinohemoproteins. However, the preference for 1-butanol of BDH is much higher than that of *C. testosteroni* EDH for ethanol or of *R. eutropha* tetrahydrofurfuryl ADH for *n*-pentanol (Geerlof *et al.*, 1994; Zarnt *et al.*, 1997). The K_m of BDH towards 1-butanol ($7 \pm 1 \mu\text{M}$) was the lowest K_m reported among other quinohemoprotein ADHs obtained from ethanol-grown *C. testosteroni* (K_m $2230 \pm 64 \mu\text{M}$ towards ethanol) (Geerlof *et al.*, 1994) or 1-butanol grown *P. putida* HK5 (K_m $105 \mu\text{M}$ towards 1-butanol) (Toyama *et al.*, 1995). Coupled with much higher K_m (K_m $535 \pm 13 \mu\text{M}$) for butyraldehyde, 1-butanol is clearly preferred over butyraldehyde by BDH. In contrast, *C. testosteroni* EDH has a higher affinity for the aldehyde (K_m for acetaldehyde is $669 \pm 22 \mu\text{M}$) such that acetic acid is produced even in the presence of ethanol.

It was recently demonstrated that butane metabolism by *P. butanovora* was mainly through a terminal oxidation pathway (Arp, 1999). Up to 90% of the butane consumed was accounted for as 1-butanol. The results of this work are consistent with terminal oxidation. The predominant ADH activity in butane-grown cells is much more efficient towards 1-butanol than 2-butanol. Furthermore, the enzyme is expressed at the highest level observed in butane-grown cells. However, butane-grown cells also expressed a low level of 2-butanol-dependent ADH activity that was not present in 1-butanol grown cells. This result may reflect a low level of subterminal butane oxidation involved in butane metabolism in

P. butanovora. Studies of the genes encoding each of these enzymes, including expression experiments and gene disruptions, will be pursued to further characterize the role of each ADH in butane metabolism.

2.6. ACKNOWLEDGMENTS

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CHAPTER 3.**TWO DISTICNT ALCOHOL DEHYDROGENASES PARTICIPATE IN
BUTANE METABOLISM BY *Pseudomonas butanovora***

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3.1 ABSTRACT

The involvement of two primary alcohol dehydrogenases, BDH and BOH, in butane utilization in *Pseudomonas butanovora* (ATCC 43655) was demonstrated. The genes coding for BOH and BDH were isolated and characterized. The deduced amino acid sequence of BOH suggests a 67-kDa alcohol dehydrogenase containing pyrroloquinoline quinone (PQQ) as cofactor and in the periplasm (29-residue leader sequence). The deduced amino acid sequence of BDH is consistent with a 70.9-kDa, soluble, periplasmic (37-residue leader sequence) alcohol dehydrogenase containing PQQ and heme c as cofactors. BOH and BDH mRNAs were induced whenever the cell's 1-butanol oxidation activity was induced. When induced with butane, the gene for BOH was expressed earlier than the gene for BDH. Insertional disruption of *bdh* or *boh* affected adversely, but did not eliminate, butane utilization by *P. butanovora*. The *P. butanovora* mutant with both genes, *boh* and *bdh*, inactivated was unable to grow on butane or 1-butanol. These cells, when grown in citrate and incubated in butane, developed butane oxidation capability and accumulated 1-butanol. The enzyme activity of BOH was characterized in cell-free extracts of the *P. butanovora* strain with *bdh* disrupted. Unlike BDH, BOH oxidized 2-butanol. The results support the involvement of two distinct NAD⁺-independent, PQQ-containing alcohol dehydrogenases, BOH (a quinoprotein) and BDH (a quinohemoprotein), in the butane oxidation pathway of *P. butanovora*.

3.2 INTRODUCTION

Pseudomonas butanovora (ATCC 43655) is an aerobic Gram negative proteobacterium closely related to the genera *Thauera* and *Azoarcus* by analysis of its 16S rRNA (Anzai *et al.*, 2000). This organism has been grouped in the genus *Pseudomonas* based on its morphology, physiology and biochemistry (Takahashi, 1980; Takahashi *et al.*, 1980). *P. butanovora* was isolated from activated sludge from an oil refining company for the purpose of generating biomass from *n*-alkanes (Takahashi, 1980; Takahashi *et al.*, 1980). *P. butanovora* can derive energy for growth from C₂ to C₉ *n*-alkanes and any of their oxidation products as well as from a variety of other carbon sources (Takahashi, 1980; Takahashi *et al.*, 1980). Butane-grown *P. butanovora* can oxidize some chlorinated hydrocarbons by cometabolism through the action of a monooxygenase (Hamamura *et al.*, 1997) and thus may have applications to bioremediation schemes.

The pathway for the oxidation of butane in *P. butanovora* proceeds primarily from butane to 1-butanol, to butyraldehyde, to butyrate (Arp, 1999) and then probably to the β -oxidation pathway of fatty acid oxidation. As in other alkane utilizers (Ashraf *et al.*, 1994; Perry, 1980; Stephens & Dalton, 1986), in *P. butanovora* the oxidation of the alkane (butane) is initiated by the action of a monooxygenase (Hamamura *et al.*, 1999). Each intermediate in the pathway accumulated in the presence of appropriate inhibitors, supported cell growth and stimulated O₂ consumption (Arp, 1999). The presence of a terminal butane oxidation pathway (i.e. production of 1-butanol) was indicated in *P. butanovora* (Arp, 1999). Although butane-grown cells consumed 2-butanol, 2-butanol production (indicative of a subterminal oxidation pathway) was not demonstrated, even in the presence of appropriate inhibitors of 2-butanol consumption. For *P. butanovora* four different alcohol dehydrogenases (ADHs) with different specificity towards primary and secondary alcohols were identified on native gels

stained for activity (Vangnai & Arp, 2001). Among these ADHs, 1-butanol dehydrogenase (BDH) was characterized biochemically (Vangnai & Arp, 2001). BDH enzyme activity was detected in butane- and 1-butanol-grown cells but not lactate-grown cells. BDH is a soluble, periplasmic, type II NAD⁺-independent quinoxinoprotein that contains 1.0 mol pyrroloquinoline quinone (PQQ) and 0.25 mol ratio heme c as prosthetic groups and exists as a monomer with an apparent molecular mass of 67 kDa (Vangnai & Arp, 2001).

The liquid-alkane metabolism of other gram-negative proteobacteria such as *Pseudomonas oleovorans*, *Pseudomonas putida* (Chakrabarty *et al.*, 1973; Eggink *et al.*, 1988; Eggink *et al.*, 1987) and *Acinetobacter* sp. strain ADP1 (Geissdorfer *et al.*, 1999; Maeng *et al.*, 1996; Ratajczak *et al.*, 1998; Ratajczak *et al.*, 1998) have been studied. From these studies and from our research with the gaseous alkane utilizer *P. butanovora*, several differences among the enzymes involved in the metabolism of alkanes are starting to emerge. First, the essential enzymes in the utilization of the alkane differ in the cellular location among these proteobacteria and *P. butanovora*. For example, the oxidation of octane in *P. oleovorans* and *Acinetobacter* sp. strain ADP1 proceeds through membrane-bound monooxygenases. In *P. butanovora* the oxidation of butane proceeds via a soluble alkane monooxygenase (Arp Laboratory, unpublished results). Second, the oxidation of the resulting alcohol in *n*-alkane metabolism proceeds via diverse enzymes depending on the bacterium. In *P. oleovorans* there is an inducible ADH which is a flavin-containing enzyme (van Beilen *et al.*, 1994) while in *P. butanovora* we described BDH, an inducible PQQ- and heme c-containing ADH (Vangnai & Arp, 2001). Constitutive ADH activity was not detected in *P. butanovora* (Sayavedra-Soto *et al.*, 2001) while *Acinetobacter* sp. strain ADP1 has at least one constitutive ADH activity (Singer & Finnerty, 1985). Third, these alkane-utilizing proteobacteria have different gene arrangements. The genes coding for the enzymes in alkane metabolism in *P. oleovorans* are clustered in an

operon (*alk*) (van Beilen *et al.*, 1994) and in *Acinetobacter* sp. strain ADP1, the genes for alkane metabolism are spread through its chromosome (Ratajczak *et al.*, 1998; Ratajczak *et al.*, 1998). The genetic arrangement of the genes for alkane metabolism in *P. butanovora* has not yet been determined. However, in *P. butanovora*, the genes for alkane metabolism may be arranged in different operons as in *Acinetobacter* sp. strain ADP1, since each enzyme activity in the butane oxidation pathway is induced independently by the substrate being oxidized (Sayavedra-Soto *et al.*, 2001).

This study suggests that the NAD⁺-independent PQQ alcohol dehydrogenase BOH (a quinoprotein) is linked to butane metabolism in conjunction with the previously characterized BDH (a quinohemoprotein; (Vangnai & Arp, 2001)). The inferred amino acid sequence of the gene coding for BOH (*boh*) showed a polypeptide similar to periplasmic PQQ-containing ADHs in other bacteria. The expression of *boh* was compared to the expression of *bdh* (encoding BDH). Inactivation of each gene coding for BOH or BDH decreased the rate of growth on butane and inactivation of both genes eliminated the growth of *P. butanovora* on butane and on 1-butanol.

3.3 MATERIALS AND METHODS

3.3.1 Cell culture and assay conditions

Cells of *Pseudomonas butanovora* (ATCC 43655) were grown in sealed serum bottles as previously described (Arp, 1999; Docker *et al.*, 1986; Takahashi *et al.*, 1980) but with the omission of yeast extract and CO₂. A headspace of at least

50 % of the total volume was used in the bottles to assure an adequate supply of O₂ to the cells. For growth with butane, the gas was added as overpressure (10% v/v of the headspace). Butane gas (99%) was purchased from Airgas, Inc. (Randor, PA). For growth in sodium lactate, the substrate was added to the sterile basal medium at concentrations of 5 to 10 mM. The bottles were incubated with shaking at 30 °C for 1 to 3 days until optical densities (600 nm) of about 0.5 were observed. All chemicals were analytical grade.

3.3.2 Activity assays

Butane monooxygenase activity was assayed by following the accumulation of ethylene oxide from ethylene, an alternative substrate for the monooxygenase (Sayavedra-Soto *et al.*, 2001). Ethylene oxidation was determined in capped serum vials (10 ml) with 20% (v/v) ethylene, 5 mM sodium butyrate and 1 ml of cell suspension (0.5 mg protein). By using a relatively high concentration of ethylene, the inactivation of the monooxygenase by ethylene oxide was prevented (Hamamura *et al.*, 1999). The accumulation of ethylene oxide was determined by injecting 100µl of the headspace into a gas chromatograph as described below.

The *in vivo* assay for 1-butanol consumption was carried out in serum vials (10 ml) with 1 mM of the substrate and 1 ml cell suspension (0.5 to 5 mg protein). The vials were capped with gray-butyl rubber stoppers and aluminum crimp seals. The capped vials were mixed by shaking in a reciprocating water bath at 30°C. Substrate consumption during the incubation was determined by injecting 5 µl of the liquid phase into a gas chromatograph as described below. Control cells not exposed to butane or 1-butanol were grown in lactate and did not have butane, ethylene or 1-butanol consumption activities.

The *in vitro* oxidations of 1-butanol and other alcohols and aldehydes were measured as phenazine methosulfate (PMS)-mediated-dichlorophenolindophenol (DCPIP) reduction. DCPIP reduction was monitored spectrophotometrically at 600 nm in a reaction mixture consisting of 25 mM MOPS (morpholinepropanesulfonic acid) at pH 7.0, 0.7 mM PMS, 0.1 mM DCPIP, 4 mM NH₄Cl, 0.8 mM KCN, 1 mM alcohol or aldehyde substrate and 0.1 to 0.5 mg of protein in a total volume of 3 ml. BOH required 1-h incubation with 5 mM PQQ for activation.

3.3.3 Analytical techniques

The concentrations of the substrates were determined by gas chromatography (GC-8A, Shimadzu Corporation, Tokyo, Japan) with the appropriate pure compounds as standards. The gas chromatograph was equipped with a flame ionization detector and a 60-cm-long by 0.1-cm ID stainless steel column packed with Porapak Q (Waters, Milford, MA). The oven temperature was 90°C for ethylene oxide and 160 °C for 1-butanol analysis.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described (Gallagher, 1999). The protein content in the cell suspensions was determined by using the bicinchoninic acid protein assay reagent as described by the manufacturer (Pierce, Rockford, IL). The samples were added directly to the protein assay reagent with 0.01% Triton X-100 (Sigma, St. Louis, MO) added to help cell lysis. Alternatively, protein determination was performed by the protein-dye binding assay as described (Bradford, 1976). Bovine serum albumin was used as a protein standard.

3.3.4 Plasmids, bacterial strains, DNA manipulations and library screening

Table 3.1 summarizes the plasmids and strains used in this work. DNA isolation, cloning, agarose gel electrophoresis and Southern hybridization were by standard protocols (Sambrook *et al.*, 1989). Total RNA was isolated by the direct addition of acid-phenol, 100 mM sodium acetate and 1% SDS to a 500- μ l cell suspension (2 to 5 mg of cell protein). The cell suspension was mixed thoroughly and centrifuged 5 min at 16000 x g. The RNA then was recovered by ethanol precipitation and dissolved in 50 μ l of diethyl pyrocarbonate treated water. The isolated total RNA was stored at -70°C until used. DNA probes were labeled by random priming using a kit (Prime-a-gene; Promega Co. Madison, WI) and [α^{32} P] dCTP (3,000 Ci/mmol; DuPont NEN products, Wilmington, DE) following the directions of the manufacturers. Northern hybridization was carried out as described (Sayavedra-Soto *et al.*, 1998). The hybridization signals were visualized and analyzed using phosphorimaging and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The genomic library of *P. butanovora* was constructed in λ -Gem11 (Promega) using *Escherichia coli* LE392 as a host and screened as described (Sambrook *et al.*, 1989). The polymerase chain reaction (PCR) was carried out using TAQ DNA polymerase (Promega) and standard protocols (Kramer & Coen, 1999). The PCR-amplified fragments were cloned into pGEM-T Easy (Promega) following the directions of the manufacturer and *E. coli* strain JM101 as host.

DNA sequencing and oligonucleotide syntheses were performed at the Central Services Laboratory of the Center for Gene Research and Biotechnology in Oregon State University. Sequence analysis was performed using software from the Wisconsin Package Version 10.0 (Genetics Computer Group (GCG), Madison, Wisc).

Table 3.1 Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
<i>E. coli</i> JM101	F', <i>traD36</i> , <i>proA</i> ⁺ , <i>proB</i> ⁺ , <i>lacI</i> ^q , <i>lacZ</i> ΔM15/ <i>supE</i> , <i>thi</i> , Δ(<i>lac-proAB</i>)	Yanish-Perron <i>et al.</i> , 1985
<i>E. coli</i> LE392	<i>hsdR514</i> , (<i>r_k</i> ⁻ , <i>m_k</i> ⁺), <i>supE44</i> , <i>supF58</i> , <i>lacY1</i> , <i>galK2</i> , <i>galT22</i> , <i>metB1</i> , <i>trpR55</i>	Murray <i>et al.</i> , 1977
<i>P. butanovora</i> ATCC 43655	Wild type strain	American Type Culture Collection
<i>P. butanovora boh::tet</i>	Mutant strain with the gene for BOH inactivated: tetracycline resistant	This work
<i>P. butanovora bdh::kan</i>	Mutant strain with the gene for BDH inactivated: kanamycin resistant	This work
<i>P. butanovora boh::tet-bdh::kan</i>	Mutant strain with the genes for BDH and BOH inactivated: tetracycline and kanamycin resistant	This work
Plasmids and λ clones		
λ Pbu1	λ clone (14 kb) with the gene for BOH	This work
pPbu1	7 kb clone containing <i>boh</i> in pBluescript II SK(+)	This work
pPbu11	pPbu1 with the <i>tet</i> cassette inserted into the <i>EcoRI</i> site in <i>boh</i>	This work
pAV1	Partial <i>bdh</i> clone (0.7 kb) into pGEM-T Easy	This work
pAV2	Subclone of λPbu5 containing <i>bdh</i> with the <i>kan</i> cassette inserted	This work
λ Pbu5	λ clone (20 kb) with the gene for BDH	This work

Table 3.1 (continued)

Strain or plasmid	Relevant characteristics	Reference or source
Cloning plasmids		
Lambda GEM-11	λ vector used to construct the genomic library of <i>P. butanovora</i>	Promega
Pbluescript II SK(+)	2,961 bp phagemid derived from pUC19; multiple cloning cassette; Amp ^r .	Genebank #X52328
pGEM-T Easy	PCR product cloning vector; multiple cloning cassette that includes flanking <i>Eco</i> RI sites; Amp ^r .	Promega

3.3.5 Peptide purification, N-terminus determination and enzyme enrichment

For the purification of the peptide of the putative aldehyde dehydrogenase, cell extracts from butane-grown *P. butanovora* were prepared by passing a cell suspension through a French Press cell disrupter at 5,000 lbs/cm². The cell extract was then subjected to ultracentrifugation (45000 x g) and the supernatant was fractionated with a 1.6- cm by 20- cm Q-Sepharose FF column (Amersham Pharmacia, Piscataway, NJ) using a gradient from 0 to 1.0 M NaCl at 1.5 ml/min. The enriched peptide (55 kDa) was separated by SDS-PAGE and then electroblotted onto polyvinylidene difluoride (PDVF) membrane (Millipore Corp., Bedford, Mass.). The N-terminal amino acid sequence of the peptide for the aldehyde dehydrogenase was determined by the Biotechnology Laboratory of the Institute of Molecular Biology at the University of Oregon. The partial BDH amino acid sequences were previously reported (Vangnai & Arp, 2001). The partial purification of BOH was performed using the *bdh::kan* mutant strain of *P. butanovora*, which lacks BDH. The soluble cell extract of the *bdh::kan* mutant strain which contained 1-butanol and PQQ-dependent DCPIP reductase activity, was purified through Q-Sepharose FF column using a NaCl linear gradient (0 to 1.0 M) in 25 mM MOPS (pH 7.0). The active BOH-containing fraction (eluted at 0.19 to 0.3 M) was concentrated with a centrifugal filter membrane (Centricon YM-30, Amicon, Millipore Corp), which removed molecules smaller than 30 kDa. The partially purified BOH was used to determine the substrate specificity of the enzyme.

3.3.6 DNA constructs and generation of the mutant strains

For the inactivation of *boh*, the tetracycline gene from pALTER-1 (Promega) with the restriction site-modified tetracycline resistance gene (*tet*) was isolated by the polymerase chain reaction (PCR) and cloned into pGEM-T Easy. The *tet* gene was then subcloned into the *Eco*RI restriction site 622 nucleotides downstream of the ATG start codon of *boh* in plasmid pPbu11 (Table 3.1). For the inactivation of *bdh*, the kit EZ::TN <KAN> from Epicentre (Madison, WI) was used to insert a transposon conferring kanamycin resistance (*kan*) into the coding region of *bdh* following the directions of the manufacturer. The insertion of the *kan* gene was localized by nucleotide sequence determination at 715 nucleotides downstream of the ATG start codon of *bdh* in plasmid pAV2. The antibiotic-resistance alcohol dehydrogenase constructs were then introduced into *P. butanovora* cells by electroporation. *P. butanovora* cells for electroporation were harvested in early stationary phase and washed three times in sterile distilled H₂O and chilled in ice. Cell electroporation was performed in an ElectroPorator (Invitrogen, Carlsbad, Calif.) in 1-mm-gap cuvettes (Invitrogen). Electroporation conditions were 1300 volts, 71 μ F and 200 Ω . In a prechilled cuvette, 120 μ l of cells (10 μ g/ml) were premixed with the plasmid construct (~0.5 μ g in 1 μ l) and pulsed. The cells were transferred to basal medium with lactate and allowed to grow under nonselective conditions for 3 h at 30°C while shaking. Cells were then challenged with tetracycline (7 μ g/ml) or kanamycin (20 μ g/ml) and plated in lactate-antibiotic plates for selection. To obtain the mutant with both genes inactivated, the mutant with *boh* inactivated was subjected to electroporation with the *bdh-kan* construct pAV2. The resultant double mutant, *boh::tet-bdh::kan*, was selected in a basal-lactate medium supplemented with tetracycline and kanamycin.

3.3.7 Nucleotide sequence accession numbers

The DNA sequences for the genes of the two 1-butanol dehydrogenase have been deposited in the Genbank database. The nucleotide sequence of *boh* has the GenBank (NCBI) accession number AF326086. The nucleotide sequence of *bdh* has the GenBank (NCBI) accession number AF355798.

3.4 RESULTS

3.4.1 Isolation of DNA fragments coding for 1-butanol dehydrogenase BOH and for 1-butanol dehydrogenase BDH

The isolation of a DNA fragment containing the gene for BOH was achieved indirectly through the isolation of a gene cluster containing an aldehyde dehydrogenase. Analysis of polypeptide patterns of cell extracts by SDS-PAGE showed a prominent 55-kDa polypeptide expressed in butane-grown cells but not in lactate-grown cells. This 55-kDa polypeptide was purified by column chromatography and its N-terminal amino acid sequence (MIYAMPGQSGAAV) was determined. Database searches showed that the amino acid sequence was similar to the N terminus of the aldehyde dehydrogenases from *Pseudomonas aeruginosa* (strain PA01) (69% identities; accession AE004625; (Stover *et al.*, 2000)) and *Alteromonas* sp. KE10 (61% identities; accession AB009654; (Maeda *et al.*, 2000)). The degenerate oligonucleotide ATG-ATH-TAY-GCN-ATG-CCN-GGN-GAR-T was synthesized and used to screen the library of *P. butanovora*. A 9-kb genomic λ clone (λ Pbu1) was isolated. A 7.1 kb *Bam*HI DNA fragment from λ Pbu1 was cloned to form pPbu1. Preliminary sequence determination of the DNA fragment in pPbu1 showed a gene cluster coding for

the aldehyde dehydrogenase (from which the oligonucleotide sequence was deduced), a transcription regulator, and an alcohol dehydrogenase (BOH in this study). Because our interest was in characterizing the alcohol dehydrogenase activity in *P. butanovora*, we focused on the determination of the nucleotide sequence of the gene coding for BOH. The deduced amino acid sequence indicated a quinoprotein, but was distinct from the sequence of BDH (see below and (Vangnai & Arp, 2001)).

To isolate a DNA fragment of the gene for BDH degenerate PCR, primers were synthesized after the two known internal amino acid sequences of BDH (MSYAPQTGLAYFPAQNIPL and KGGGIPNLGYSTAETIAHLDQFVFK; (Vangnai & Arp, 2001)). The degenerate PCR primers (forward primer: 5' ATG AGC TAC GCC CCA CAG ACC GGC CTG GCC TAC TTY CCN GCN CAR AAY ATH TTY YT 3' and reverse primer: 5' TT CAA GAC CAA CTG GTC CAG ATG CGC GAT GGT CTC CGC GGT GCT RTA NCC NAR RTT NGG DAT NCC 3') were designed using the consensus-degenerate hybrid oligonucleotide primer approach (CODEHOP; (Rose *et al.*, 1998)). A 0.7-kb DNA fragment of the gene for BDH was amplified and cloned into pGem-T Easy to form pAV1. The nucleotide sequence of the DNA fragment in pAV1 was determined and showed similarity to other PQQ-containing alcohol dehydrogenases. This DNA fragment was used to screen the genomic library of *P. butanovora* for the gene of BDH. The genomic clone λ Pbu5, with a 20-kb, insert hybridized to the probe, was used to obtain the complete sequence of the gene coding for BDH. The nucleotide sequence of *bdh* was determined directly in the λ Pbu5 genomic clone. These results were the first indication of the presence of two quinoprotein ADHs in *P. butanovora*.

3.4.2 Analysis of the nucleotide sequences for BOH and BDH

A summary of the sequence comparisons of *boh* and *bdh* to other bacterial ADHs is shown in Table 3.2. The ORF of *boh* is composed of 1872 nucleotides. A clear start codon for *boh* was found with a Shine-Dalgarno-like ribosome binding site sequence (GGAG) 5 bases upstream. A nucleotide sequence that started 96 bases upstream of the start codon was indicative of a σ^{54} -dependent promoter (CTG GCA CGC TCT TTG CCA) in *boh* as well. The nucleotide sequence of this putative σ^{54} -dependent promoter in *P. butanovora* has 83% identity with the consensus sequence for σ^{54} -dependent promoters (Morett & Segovia, 1993). The deduced amino acid sequence of *boh* indicates a type I NAD⁺-independent ADH such as the type I quinoprotein ethanol dehydrogenase from *Pseudomonas aeruginosa* (Accession 10120672; (Diehl *et al.*, 1998)), a homodimer with subunits of relative molecular mass of 60 kDa. The calculated molecular mass of the complete polypeptide encoded by *boh* is 67,553 Da. The BOH polypeptide without the putative 29 amino-acid leader sequence has a calculated molecular mass of 64,666 Da and a pI of 6.15 is indicated.

The nucleotide sequence and the requirement of PQQ in the activity assay of BOH suggest that PQQ is the prosthetic group of BOH (see below). The ORF of *bdh* is composed of 2,078 nucleotides having a theoretical molecular mass of 70.9 kDa, compared to an experimental mass determination of 66 kDa (Vangnai & Arp, 2001). The experimental mass determination is in close agreement with a putative leader sequence of 37 residues in BDH (4.1 kDa). The calculated pI is 6.66. A Shine-Dalgarno-like ribosome binding site sequence (GGAG) is localized 6 bases upstream of the start codon. The amino acid sequence deduced from *bdh* is similar to other quinohemoproteins (Table 3.2; (Stoorvogel *et al.*, 1996)).

Upstream of the ORF coding for BDH, an ORF of 929 nucleotides with no clear Shine-Dalgarno-like ribosome binding site sequence is present. The deduced amino acid sequence has 43% similarity to that of orf1 in the *ntn* gene cluster of *Pseudomonas* sp. strain (James *et al.*, 2000) and 40% similarity to ChnX encoded in a gene cluster for cyclohexanol oxidation in *Acinetobacter* sp. strain SE19 (Cheng *et al.*, 2000), both of which have ORFs unknown function. The nucleotide sequence CTG GCA TGG CTT CTG CA is located 163 nucleotides from the putative start codon of this unknown ORF in *P. butanovora*. This sequence has 82% identity to the consensus sequence of a σ^{54} -dependent promoter (CTG GCA CGG CCT TTG CA (Morett & Segovia, 1993)).

BDH and BOH have 7 Trp residues in positions equivalent to those implied in the formation of the β -propeller fold of PQQ-containing alcohol dehydrogenases (Oubrie *et al.*, 1999; Xia *et al.*, 1996). In BOH the Trp at positions 345 (9 residues form the consensus position) and 438 (16 residues from the consensus position) offer alternatives for the Trp residues in the W7 and W8 β -propeller folds of the methanol dehydrogenase from *Methylophilus* W3A1 (Xia *et al.*, 1996). The contiguous Cys residues implied in the interaction with PQQ and Ca^{++} (Xia *et al.*, 1996) were located at positions 134 and 135 of BOH and position 130 and 131 of BDH. Comparison of the deduced amino acid sequence of BOH and the deduced amino acid sequence of BDH show 35% identities and 47% similarity (Table 3.2).

Table 3.2 Sequence comparison of BOH, BDH and other PQQ-containing alcohol dehydrogenases

PQQ-containing ADH	Organism	No. of amino acids	% identity (% similarity) to BOH	% Identity (% similarity) to BDH	Accession number
BOH	<i>P. butanovora</i>	623	35 (47)	-	AF326086
BDH	<i>P. butanovora</i>	691	-	35 (47)	AF355798
Methanol dehydrogenase	<i>Methylophylus methylotrophus</i> W3A1	573	33 (46)	30 (43)	19442860
Methanol dehydrogenase	<i>Methylobacterium exotorquens</i>	626	34 (46)	29 (42)	AAA25380
Ethanol dehydrogenase	<i>Pseudomonas aeruginosa</i>	623	70 (80)	35 (49)	CAA08896
Ethanol dehydrogenase	<i>Comamonas testosteroni</i>	708	36 (50)	60 (70)	CAA57464
Tetrahydrofurfuryl ADH	<i>Ralstonia eutropha</i>	698	37 (51)	60 (72)	AAF86335

3.4.3 Time course of *boh* and *bdh* expression upon exposure to butane

To corroborate and test the involvement of BOH and BDH in butane metabolism, lactate-grown cells were washed and incubated in basal medium plus butane. In a time course experiment, we monitored the development of ethylene oxidation activity (butane monooxygenase activity) and 1-butanol consumption. A typical induction of the butane monooxygenase and 1-butanol oxidation activities by butane was observed ((Sayavedra-Soto *et al.*, 2001), Fig. 3.1A). During the induction by butane, cell samples were withdrawn at intervals and their total RNA was extracted and blotted onto nylon membranes for analysis by northern hybridization. The probe for *bdh* was derived from the plasmid pAV1. The probe for *boh* was generated by PCR using primers that flanked the ORF for *boh* and pPbu1 as template. The probe for the 16S ribosomal RNA (16S rRNA) was obtained by PCR using eubacterial universal primers (Giovannoni, 1991) and genomic DNA as template. The 16S rRNA probe was used to show that equivalent mRNA amounts were loaded into the gel for each treatment. The same membrane blot was hybridized to the three probes separately after stripping the membrane (Fig. 3.1B). Cells exposed to butane first showed induction of *boh* in about 30 min followed by the induction of *bdh*, which was first detected at 60 min (Fig. 3.1B). At 240 min of incubation, a decrease of the total amount of mRNA for both ADHs was observed. This decrease of mRNA levels occurred as the maximum 1-butanol oxidation activity was reached (Fig. 3.1A). These results suggest that BOH and BDH are involved in butane metabolism. Furthermore, in the conditions of this study, BOH and BDH were differentially expressed upon exposure to butane. We also measured 1-butanol consumption and BOH and BDH mRNA levels during cell growth on butane. The specific activity for 1-butanol consumption increased as the cell mass of *P. butanovora* increased, reaching a maximum of $68 \text{ nmol} \cdot \text{min}^{-1} \cdot (\text{mg protein}^{-1})$ at early stationary phase (optical density at 600 nm, ~ 0.55) at 35 h

(not shown). Specific activity of 1-butanol consumption in butane-grown cells typically reaches 50 to 100 $\text{nmol}\cdot\text{min}^{-1}\cdot(\text{mg protein}^{-1})$ at optical densities of 0.5 to 0.7 (Sayavedra-Soto *et al.*, 2001). The mRNA levels of BDH and BOH were determined by using northern hybridizations. The mRNA levels of BDH and BOH were determined. Both genes were expressed, and their mRNA levels increased until the cells reached stationary phase. We were not able to detect clear differences between the patterns of *boh* and *bdh* expression during growth on butane (not shown).

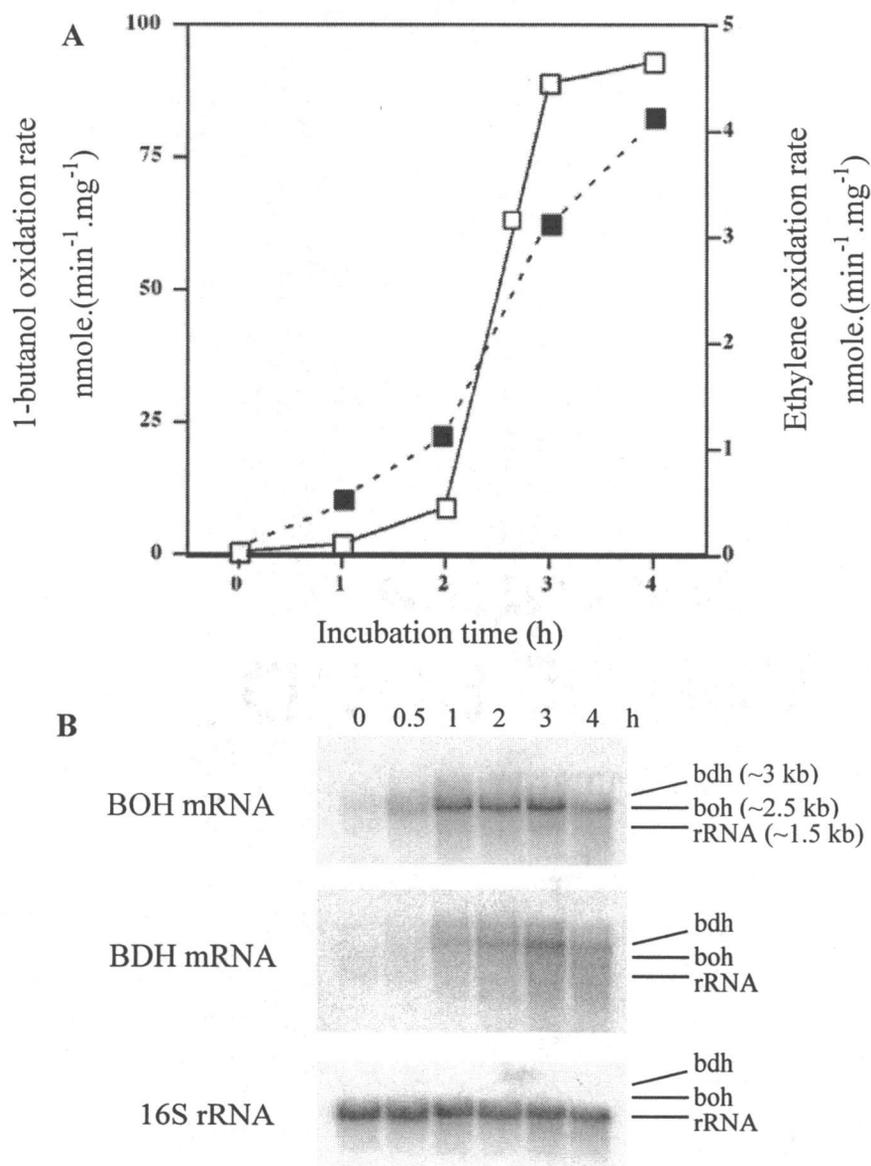


Figure 3.1 Induction of BOH and BDH total activity and mRNAs by butane. **A)** Development of ethylene oxidation (dashed line, solid squares) and 1-butanol oxidation (solid line, open squares) activities. Lactate-grown cells were washed and then incubated in basal medium with butane for the indicated times. **B)** BOH and BDH mRNA levels during the induction of butane oxidation. The same blot was used for the three hybridizations after probe stripping. The three arrows represent the relative position of each mRNA with respect to the other two. The numbers in the first frame are the estimated sizes of the mRNAs and of the 16S rRNA.

3.4.4 BOH and BDH mRNA induction by various alcohols

The DNA probes derived from the gene sequences also permitted us to determine the levels of induction of the mRNAs of BOH and BDH in response to various alcohols and butane. Lactate-grown cells were washed then exposed to basal medium containing 2 mM ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, or 1-pentanol. After 2 h of incubation in a given alcohol, the cells were harvested and their total RNA was extracted. The same membrane blot was hybridized with probes for the mRNAs of *bdh* and *boh*, and the 16S rRNA separately after probe stripping (Fig. 3.2). In these experiments the cells showing induction of BOH or BDH mRNAs also showed 1-butanol consumption (10 to 30 nmol·min⁻¹·(mg protein⁻¹)). Although the relative levels of the BOH and BDH mRNAs varied among replicate incubations, the trends were consistent. Primary C₂ and C₄ alcohols were the most effective inducers of *boh* and *bdh*. Some differences in the levels of BOH and BDH mRNAs produced were also observed when cells were exposed to C₂ to C₅ alcohols (Fig. 3.2). For example, 2-butanol is as effective as 1-butanol in inducing *boh* expression, but 2-butanol is much less effective than 1-butanol in inducing *bdh*. Compared to BDH mRNA, BOH mRNA was induced by a wider range of alcohols.

3.4.5 Gene inactivation of BOH and BDH

To address the involvement of BOH and BDH separately in the metabolism of butane, the genes for BOH and BDH were inactivated by insertion mutagenesis. The tetracycline resistance cassette inserted into *boh* (pPbu11) and the kanamycin resistance cassette inserted into *bdh* (pAV2) were introduced by electroporation into *P. butanovora* producing the mutant strains *boh::tet* and *bdh::kan* respectively.

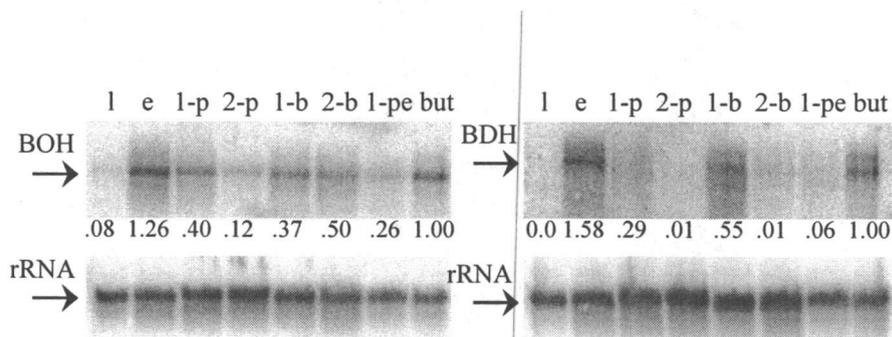


Figure 3.2 Induction of the mRNA for BOH and BDH upon incubation with different alcohols and butane. The same RNA preparation was probed for the presence of the BOH and BDH mRNAs. For comparison among treatments, the blots were stripped and hybridized to a probe for the 16S rRNA. Lactate grown cells were washed and then incubated for 2 h in medium containing the indicated substrate and then tested for the presence of BOH and BDH mRNAs by northern hybridization. Cells were incubated with lactate (l), ethanol (e), 1-propanol (1-p), 2-propanol (2-p), 1-butanol (1-b), 2-butanol (2-b), 1-pentanol (1-pe), and butane (but). The numbers above the 16S rRNA blot were calculated from two batches of cells and are the ratios of the BOH or BDH mRNA signal to the rRNA signal normalized to the ratios obtained with cells exposed to butane.

The mutant strain with both *boh* and *bdh* inactivated was also produced (*boh::tet-bdh::kan*). The mutations were confirmed with probes for *boh*, *bdh* or for the antibiotic markers in Southern hybridizations (Fig. 3.3), where the sizes of the restriction fragments containing each gene were increased by the sizes of the antibiotic markers. Growth on butane was delayed when *boh* or *bdh* was inactivated. After a lag period of about 12 h both mutant cells began to grow and eventually reached similar optical densities as those observed in the wild-type cells. The lack of BOH slowed cell growth more than the lack of BDH (Fig. 3.4). Cells with *boh* or *bdh* inactivated growing on 1-butanol reached final optical densities that were only half of that observed in the wild-type (Fig. 3.4). When both genes were inactivated, growth on butane and 1-butanol was eliminated (Fig. 3.4). Citrate-grown cells of the *boh::tet-bdh::kan* strain accumulated 1-butanol (102.5 ± 10.6 nmole) when incubated for 4.5 hours with 20% butane indicating induction of the monooxygenase.

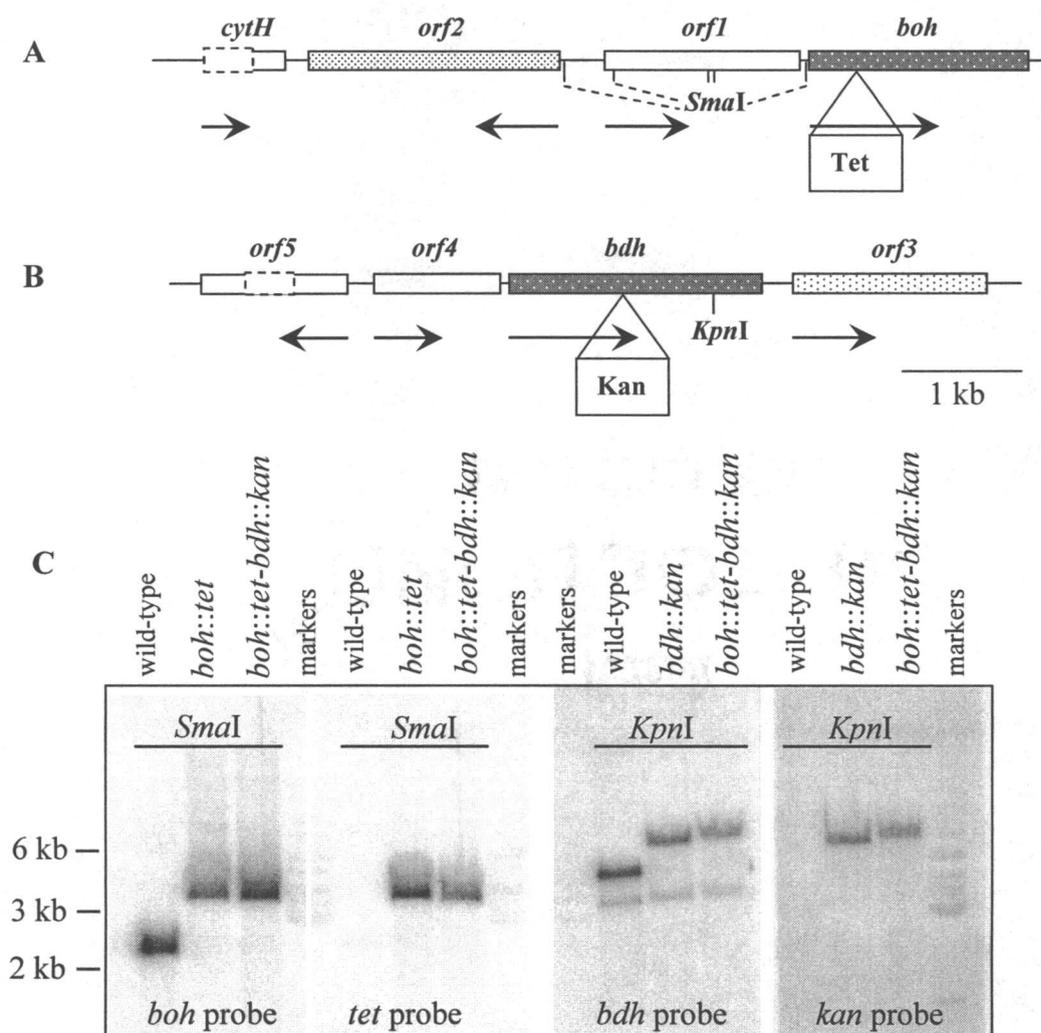


Figure 3.3 Map of the loci of *boh* (A), *bdh* (B) and phosphoimage of the Southern blot of DNA (C) from the wild-type and mutant strains of *P. butanovora*. The maps show the locations of the adjacent genes and the site of insertion of the antibiotic-conferring cassettes. The arrows under the genes show the direction of transcription. The nucleotide sequences of the genes adjacent to the alcohol dehydrogenase-encoding genes are incomplete but show similarity to a regulatory element (*orf1*) and to genes coding for aldehyde dehydrogenase (*orf2*), to another aldehyde dehydrogenase (*orf3*), to an *orf* of unknown function (*orf4*) and to a regulatory element (*orf5*). The dashed lines represent undetermined sequences. In the Southern hybridization two restriction digests were used for clarity to show the different loci of *boh* and *bdh* and the increase in size as a result of the cassette insertion. The strain, restriction enzyme and the probe used are indicated.

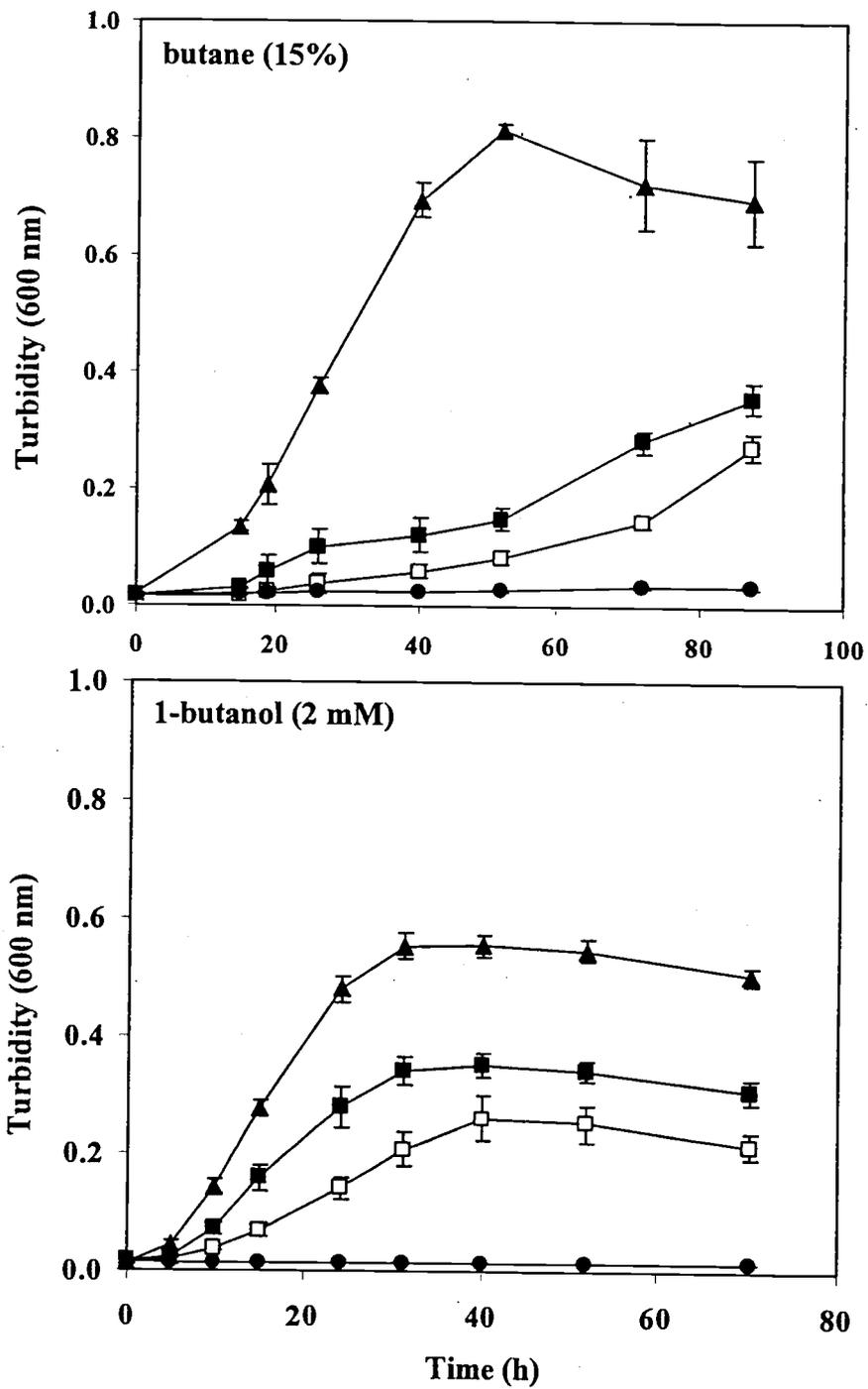


Figure 3.4 Growth of the wild-type, *boh::tet* mutant, *bdh::kan* mutant, and *boh::tet-bdh::kan* mutant strains of *P. butanovora*. The growth substrates were butane and 1-butanol as indicated. Symbols: ▲, wild-type; □, *boh::tet*; ■, *bdh::kan*; and ●, *boh::tet-bdh::kan* *P. butanovora* strains.

3.4.6 Biochemical characterization of BOH

The *bdh::kan* mutant strain facilitated the characterization of BOH in cell extracts. Quinoproteins lacking heme c (i.e. methanol dehydrogenase and ethanol dehydrogenase (EDH) from *P. aeruginosa* and *P. putida* and soluble glucose dehydrogenase from *E. coli*) do not react with ferricyanide (Docker *et al.*, 1986; Geerlof *et al.*, 1994; Groen *et al.*, 1984); thus, ferricyanide reductase activity in the *bdh::kan* mutant strain should be absent. As expected, cell extract of the mutant strain lacking BDH showed $<2 \text{ nmol} \cdot \text{min}^{-1} \cdot (\text{mg protein})^{-1}$ ferricyanide reductase activity towards 1-butanol, a fraction of that typically observed in the wild-type *P. butanovora* ($102 \pm 19.6 \text{ nmol} \cdot \text{min}^{-1} \cdot (\text{mg protein})^{-1}$). BOH was partially purified (3.4-fold increase in specific activity) from the soluble cell extract of butane-grown *bdh::kan* mutant strain. Following activation with PQQ, this BOH preparation was used to examine the substrate range of BOH (Table 3.3). As expected 1-butanol was a good substrate. However, 2-butanol supported 15 % higher rates of activity. In contrast, BDH does not oxidize 2-butanol (Vangnai & Arp, 2001). BOH also exhibited slightly higher activity with 2-propanol and 2-pentanol relative to 1-propanol and 1-pentanol (Table 3.3). The partially purified BOH also oxidized butyraldehyde, the product of 1-butanol oxidation (Table 3.3). This PMS-mediated butyraldehyde oxidation activity was not likely due to another enzyme in the partially purified BOH preparation because cells lacking both BOH and BDH (*boh::tet-bdh::kan* mutant strain) did not show this activity, even when incubated with 1-butanol or butyraldehyde for 6 h to induce activity.

Table 3.3 Specific activity^a of BOH towards primary alcohols, secondary alcohols and aldehydes.

Substrate	Mean \pm SD of specific activity [nmol \cdot min ⁻¹ \cdot (mg of protein) ⁻¹]	Relative activity (%)
Methanol	2 \pm 0.5	0.7
Ethanol	82 \pm 12	30
1-Propanol	61 \pm 7	22
1-Butanol	239 \pm 17	87
1-Pentanol	92 \pm 10	34
1-Octanol	59 \pm 10	22
2-Propanol	84 \pm 9	30
2-Butanol	274 \pm 9	100
2-Pentanol	167 \pm 6	61
2-Octanol	42 \pm 1	15
Propionaldehyde	91 \pm 5	33
Butyraldehyde	68 \pm 22	25

^aPMS-mediated, DCPIP reduction activity of the partially purified BOH (see text).

3.5 DISCUSSION

In this study, we show that *P. butanovora* has two distinct genes (*boh* and *bdh*) encoding two PQQ-containing alcohol dehydrogenases (a quinoprotein and a quinohemoprotein) and that both alcohol dehydrogenases participate in butane metabolism. The nucleotide sequences of the genes and Southern hybridizations argue for different genetic loci for *boh* and *bdh*. Northern analyses and gene inactivation experiments link each gene to the metabolism of butane. The involvement of *boh* and *bdh* in butane metabolism comes from several lines of evidence. First, neither of the genes is expressed in lactate-grown cells (which are devoid of butane metabolism). Second, both genes are expressed in butane-grown

cells. Third, when lactate- or citrate- grown cells are washed and subsequently exposed to butane, both genes are expressed as cells develop butane and 1-butanol oxidation activities. Fourth, gene inactivation experiments, where either gene was inactivated, showed that *P. butanovora* grew more slowly on butane compared to the wild-type strain. When both *boh* and *bdh* were inactivated, growth on butane and 1-butanol was eliminated. The involvement of BDH in the butane oxidation pathway in *P. butanovora* was previously established at the biochemical and physiological levels as well (Vangnai & Arp, 2001).

The involvement of diverse alcohol dehydrogenases in alcohol or alkane metabolism is not without precedent (Toyama *et al.*, 1995; Van Der Linden & Huybregtse, 1969). For example, *Pseudomonas putida* produces three distinct PQQ-containing ADHs (a quinoprotein and two quinohemoproteins) each with different substrate ranges, and each induced primarily by a different alcohol (Toyama *et al.*, 1995). *Rhodococcus rhodochrous* PNKb1 produces two distinct NAD⁺-dependent ADHs, one for primary and another for secondary alcohols, and each is required for the metabolism of propane (Ashraf & Murrell, 1992). However, the situation with *P. butanovora* seems to be different because genes for two distinct PQQ-containing alcohol dehydrogenases were expressed in response to 1-butanol which was generated in the oxidation of butane.

To gain additional insights to the role of each of these ADHs in *P. butanovora*, we investigated the expression of each gene in response to different alcohols (C₂-C₅) and butane. The highest levels of mRNA for both BOH and BDH were found in cells exposed to ethanol, 1-butanol and butane. Differences in the expression patterns were also apparent. For example, compared to *bdh*, *boh* was induced by a broader range of alcohols, and higher levels of mRNA for BOH were induced in the presence of 1-propanol and 2-butanol than for BDH (Fig. 3.3). The induction patterns reflected the substrate ranges of each enzyme. The induction of

boh expression by 2-butanol and the higher specific activity of BOH for 2-butanol suggest a possible role for BOH. Perhaps BOH oxidizes both 1- and 2-butanol, both of which are potential products of butane oxidation. While 1-butanol was shown to be the predominant product, production of a low level of the subterminal oxidation product, 2-butanol, was not demonstrated (Arp, 1999). The deduced amino acid sequence of BOH is 80% similar to that of the quinoprotein ethanol dehydrogenase (EDH) from *P. aeruginosa* which also exhibits a wide substrate range. In addition to ethanol, EDH oxidizes both primary (1-propanol and 1-butanol) and secondary (2-propanol and 2-butanol) alcohols efficiently (Rupp & Gorisch, 1988). BOH oxidizes 1-butanol and 2-butanol efficiently (Table 3.3). In contrast, BDH, which is only 30% similar with EDH, efficiently oxidizes 1-propanol and 1-butanol, but not 2-propanol or 2-butanol (Vangnai & Arp, 2001). The deduced amino acid sequence of BDH shows 72% similarity to quinohemoprotein tetrahydrofurfuryl ADH of *Ralstonia eutropha*. Tetrahydrofurfuryl ADH and BDH have high activity towards 1-butanol and 2-pentanol, but little or no activity with 2-butanol (Vangnai & Arp, 2001; Zarnt *et al.*, 1997).

The lack of growth of the *boh::tet-bdh::kan* mutant strain on either butane or 1-butanol suggests that there are only two primary alcohol dehydrogenases involved in the butane metabolism of *P. butanovora*. Furthermore, citrate grown *boh::tet-bdh::kan* cells when incubated in butane tended to accumulate 1-butanol reinforcing the notion of only two primary alcohol dehydrogenases. Perhaps the presence in *P. butanovora* of these two distinct ADHs that are induced in the butane oxidation pathway reflects the need of the cells to treat 1-butanol as both a source of energy and as a toxic compound. One ADH may respond to low levels of 1-butanol as a metabolite and the second ADH may respond to higher levels of 1-butanol as a toxin. Cells of the *boh::tet-bdh::kan* mutant strain did die when incubated in 1-butanol for extended periods. Another possible reason for the two

distinct ADHs induced by the same substrate is that of specialization for different bioenergetic roles. BOH (the quinoprotein) is expected to require a cytochrome as the immediate acceptor which can then transfer electrons to the respiratory chain (Goodwin & Anthony, 1998). BDH, with a heme as a prosthetic group, could transfer electrons to azurin as in the case with the quinoprotein of *P. putida* (Toyama *et al.*, 2001).

This work demonstrates the involvement of two similar alcohol dehydrogenases for the oxidation of 1-butanol in the butane metabolism of *P. butanovora*. In addition we show that BOH and BDH have different substrate specificity and that their mRNAs are expressed in response to similar stimuli but to different extents. The reasons for the existence of this dual pathway in *P. butanovora* awaits further experimentation into bioenergetics and toxicity of 1-butanol.

3.6 ACKNOWLEDGMENTS

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CHAPTER 4.**ROLES FOR THE TWO 1-BUTANOL DEHYDROGENASES OF
Pseudomonas butanovora
IN BUTANE AND 1-BUTANOL METABOLISM**

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4.1 ABSTRACT

Pseudomonas butanovora grown on butane or 1-butanol expresses two 1-butanol dehydrogenases, a quinoprotein (BOH) and a quinohemoprotein (BDH). BOH exhibited high preference towards 1-butanol ($K_m = 1.7 \pm 0.2 \mu\text{M}$). BOH also oxidized butyraldehyde and 2-butanol ($K_m = 369 \pm 85 \mu\text{M}$ and $662 \pm 98 \mu\text{M}$, respectively). The mRNA induction profiles of BOH and BDH at three different levels of 1-butanol, a non-toxic level (0.1 mM), a growth-supporting level (2 mM) and a toxic level (40 mM) were similar. When the citrate-containing growth medium contained different levels of 1-butanol, wild-type *P. butanovora* could tolerate higher levels of 1-butanol than the mutants (the *boh::tet* strain and the *bdh::kan* strain), i.e. 1-butanol concentrations up to 40 mM enhanced growth of the wild-type cells while concentrations higher than 10 mM became toxic to both single mutants. A model is proposed where the electrons from 1-butanol oxidation follow a branched electron transport chain. BOH may be coupled to ubiquinone with the electrons being transported to a cyanide-sensitive terminal oxidase. In contrast, electrons from BDH may be transferred to a terminal oxidase that is less sensitive to cyanide. The former pathway may function primarily in energy generation while the latter may be more important in the detoxification of 1-butanol.

4.2 INTRODUCTION

Pseudomonas butanovora (ATCC 43655) is a Gram negative, rod-shaped bacterium that was isolated from activated sludge from an oil refining plant using *n*-butane as the energy source (Takahashi *et al.*, 1980). *P. butanovora* can utilize

a variety of organic compounds as the growth substrate: C₂-C₉ *n*-alkanes, the corresponding primary alcohols, carboxylic acids and some polyvalent alcohols, but not alkenes, sugars or C₁ compounds (Takahashi, 1980; Takahashi *et al.*, 1980). Butane-grown *P. butanovora* can degrade some chlorinated aliphatic hydrocarbons (Hamamura *et al.*, 1997), and thus has potential in the bioremediation of sites contaminated with these solvents. The pathway of butane metabolism by butane-grown *P. butanovora* was determined to follow the terminal oxidation pathway, that is butane → 1-butanol → butyraldehyde → butyrate (Arp, 1999).

Alcohol metabolism has been studied in both alkane and alcohol grown bacteria. For example, alcohol dehydrogenases (ADHs) induced in propane-grown *Rhodococcus rhodochrous* PNKb1, *Mycobacterium vaccae* JOB5 and *Pseudomonas fluorescens* NRRL B-1244 were purified and characterized as NAD⁺-dependent secondary ADH(s) (Ashraf & Murrell, 1990; Ashraf & Murrell, 1992; Coleman & Perry, 1985). In *R. rhodochrous* PNKb1, NAD⁺-dependent alcohol dehydrogenase activities specific for either 1-propanol or 2-propanol were demonstrated (Ashraf & Murrell, 1992). Multiple ADHs have been described in alkane-utilizing and alcohol-utilizing bacteria. Multiple NAD⁺- and NADP⁺-dependent ADHs were also demonstrated in *Acinetobacter* sp. strain HO1-N. ADH-A was required for growth on ethanol and short-chain alcohols, an ADH-B was specified for mid-chain length alcohols, and a hexadecanol dehydrogenase (HDH) was induced specifically under growth on hexadecane and hexadecanol (Singer & Finnerty, 1985).

Some ADHs involved in alkane and alcohol metabolism do not couple to NAD(P)⁺ and contain pyrroloquinoline quinone (PQQ) as the prosthetic group. For example, methanol dehydrogenase (MDH) in methylotrophic bacteria was the first enzyme shown to contain a PQQ as the prosthetic group (Anthony, 1993). The physiological electron acceptor for MDH is a specific *c*-type cytochrome, cytochrome *c_L*, which then is oxidized by cytochrome *c_H* (Afolabi *et al.*, 2001;

Anthony, 1998). The terminal oxidase in methylotrophic bacteria is either a cytochrome *aa*₃ or a cytochrome *co* depending on the type of organism and growth conditions (Anthony, 1993). In other oxidative nonmethylotrophic bacteria, ADHs have been classified into three groups (type I, II, and III) according to their molecular properties, catalytic properties and localization (Matsushita *et al.*, 1994). The molecular structure of type I ADH found in *Pseudomonas aeruginosa* and *Pseudomonas putida* (Gorisch & Rupp, 1989; Groen *et al.*, 1984; Toyama *et al.*, 1995) resembles that of MDH, but has very low affinity for methanol. Type I ADH uses a *c*-type cytochrome (cytochrome *c*_{QEDH} or cytochrome *c*₅₅₀) as the electron acceptor (Schobert & Gorisch, 1999; Schrover *et al.*, 1993) which subsequently reacts with another *c*-type cytochrome or copper-containing protein (azurin) (Anthony, 1998; Matsushita *et al.*, 2000). Type II ADHs are soluble periplasmic quinoxinoproteins, having PQQ and heme *c* as prosthetic groups and have been found in *Comamonas testosteroni* (De Jong *et al.*, 1995; Groen *et al.*, 1986), *P. putida* (Toyama *et al.*, 1995), and *Ralstonia eutropha* (Zarnt *et al.*, 1997). *P. putida* HK5 when grown on ethanol, 1-butanol and 1,2-propanediol produces three different quinoprotein ADHs: one type I ADH and two type II ADHs (ADH IIB and ADH IIG), respectively (Toyama *et al.*, 1995). Type III ADHs are membrane-associated enzymes found in the cytoplasmic membrane of acetic acid bacteria. Type III ADHs have three subunits: a quinoxinoprotein, a triheme cytochrome *c* and third subunit of unknown function (Adachi *et al.*, 1978; Matsushita *et al.*, 1994). The electron acceptor of type III ADHs is ubiquinone (Anthony, 1998; Matsushita *et al.*, 1996).

In butane-grown *P. butanovora*, five different ADHs with different specificity towards primary and secondary alcohols were identified (Vangnai & Arp, 2001; Vangnai *et al.*, 2002). Among these, *P. butanovora* expresses two distinct NAD⁺ dependent PQQ-containing 1-butanol dehydrogenases, BOH (a quinoprotein) and BDH (a quinoxinoprotein). The substrate range of BOH and its

gene were characterized (Vangnai *et al.*, 2002). BOH is a 64-kDa type I quinoprotein without its putative 29-residue leader sequence and is located in the periplasm. BDH was characterized biochemically and genetically (Vangnai & Arp, 2001; Vangnai *et al.*, 2002). BDH is a soluble, periplasmic, type II quinohemoprotein that contains 1.0 mol (PQQ) and 0.25 mole ratio heme c as prosthetic groups and exists as a monomer with an apparent molecular mass of 67 kDa (Vangnai & Arp, 2001). When the gene coding for either BOH or BDH was inactivated, the mutant cells (the *boh::tet* strain and the *bdh::kan* strain) were still able to grow on butane and 1-butanol. The growth rate of both mutant strains on butane was decreased, but eventually they reached similar optical densities as that observed in wild-type cells. Growth of the mutant strains on 1-butanol reached final densities that were half of that observed in wild-type cells, but the growth rates of each mutant on butane and 1-butanol were similar. Growth on butane and 1-butanol was eliminated when the genes for both BOH and BDH were inactivated, which demonstrates their essential role in the butane and 1-butanol oxidation pathway (Vangnai *et al.*, 2002). However, these previous studies have not revealed why *P. butanovora* needs two 1-butanol dehydrogenases. Our goal was to elucidate the roles of BOH and BDH in butane and 1-butanol metabolism in *P. butanovora*. We established the kinetic characteristics of BOH and BDH and the possible functions in 1-butanol detoxification. The expression patterns of the genes coding for each enzyme in response to different levels of 1-butanol are also described. Two distinct electron transport systems used by BOH and BDH and a schematic model for 1-butanol-dependent respiratory systems in *P. butanovora* are proposed.

4.3 MATERIALS AND METHODS

4.3.1 Cell culture and chemicals

Cells of *Pseudomonas butanovora* were grown in sealed serum bottles (150 ml) as previously described (Takahashi, 1980) but with the omission of yeast extract and CO₂. A headspace of at least 50 % of the total volume was used in the bottles to assure an adequate supply of O₂ to the cells. For growth with 1-butanol, the substrate was added into the medium to a final concentration of 2 mM. For growth in sodium lactate or sodium citrate, each substrate was added to the sterile basal medium at concentrations of 2 to 10 mM. The bottles were incubated with shaking at 30°C for 1 to 3 days until optical densities (600 nm) of about 0.5 were observed.

Wild-type *P. butanovora* was obtained from the American Type Culture Collection (ATCC 43655). The *boh::tet* strain has the *boh* gene inactivated by the insertion of tetracycline-resistant (*tet*) gene at the *EcoRI* restriction site, 622 nucleotides downstream of the ATG start codon of *boh*. The *bdh::kan* strain has the *bdh* gene inactivated by the insertion of a transposon conferring a kanamycin-resistant (*kan*) gene at 715 nucleotides downstream of ATG start codon of *bdh* (Vangnai *et al.*, 2002).

Alcohols and aldehydes (98%-99.99%) were purchased from Sigma and Aldrich (St. Louis, Mo.). Other chemicals used were analytical grade.

4.3.2 Preparation of soluble fraction of *bdh::kan* mutant strain and purification of BOH

BOH was purified from the soluble fraction prepared from 1-butanol-grown *bdh::kan* mutant strain. Cells of *bdh::kan* strain grown on 2-4 mM 1-butanol were harvested at an OD₆₀₀ of 0.5, washed twice and resuspended to 1-1.5 g/ml in 25 mM MOPS (morpholinepropanesulfonic acid) buffer pH 7.0. The cell suspension was frozen at -20°C for at least for 24 h, then thawed at room temperature. For cell lysate preparation, lysozyme (0.2 mg/ml), a small amount of DNA nuclease I and MgSO₄ (final concentration of 2 mM) were added to the cell suspension. The mixture was gently homogenized by a pre-cooled glass homogenizer and then gently rocked at room temperature for 1 h. Unbroken cells were removed by 15-min centrifugation at 11,000 x *g* at 4°C. The membrane fraction was separated by centrifugation at 200,000 x *g* (SW40, Beckman L8-70) for 1 h at 4°C. The supernatant containing BOH was kept at -80°C until used.

BOH was partially purified by the following steps. In each step, all fractions or pool of fractions, pre-incubated with PQQ on ice for 1 h, were examined for 1-butanol dependent phenazine methosulfate (PMS) reductase activity, followed by SDS-PAGE analysis. The PMS reductase activity was measured spectrophotometrically as described (Vangnai & Arp, 2001), but with 25 mM MOPS buffer pH 7.0. Active fractions were pooled and dialyzed against 25 mM MOPS buffer (pH 7.0) at 4°C overnight before being applied to a next column. (i) Q-Sepharose (Sigma) FPLC: the cell supernatant of *bdh::kan* mutant was applied to Q-Sepharose anion exchange FPLC column (Millipore Corp.) (2.2x18 cm) which had been equilibrated with 25 mM MOPS buffer, pH 7.0. The proteins were eluted by a linear gradient of 0 to 1 M NaCl in the same buffer. BOH eluted at 190-300 mM NaCl, (ii) Superose 6 FPLC: The active Q-Sepharose fractions were pooled, dialyzed, and then applied to Superose 6 (Sigma) gel

filtration column (1.1x80 cm). (iii) Q-Sepharose FPLC: active fractions from Superose 6 were pooled and concentrated by a centrifugal filter membrane, Centricon YM30 (Amicon, Millipore Corp.), which removed molecules smaller than 30 kDa before being applied on to Q-Sepharose column (2.2x10 cm). The proteins were eluted by continuous-step gradient of 0 to 80 mM NaCl (1 column volume, CV), 80-350 mM NaCl (15 CV), 350-400 mM (1CV) and 400 mM-1M NaCl. Active fractions from the second Q-Sepharose column, eluted at 80-350 mM NaCl, were then pooled and concentrated by a centrifugal filter membrane, Centricon YM50 (Amicon, Millipore Corp.), which removed molecules smaller than 50 kDa. The partially purified BOH was used to determine the K_m of the enzyme. The K_m (s) were calculated from the initial rates determined with the PMS reductase standard assay previously described (Vangnai & Arp, 2001) by varying the concentrations of substrates tested from 0.1 μ M-1 mM. The protein concentration was fixed at 60 nM.

4.3.3 Northern hybridization

Wild-type cells were grown on 4 mM lactate overnight. Then cells were harvested and washed twice to remove residual lactate. Cells were resuspended into the same volume (1 liter) and divided for induction experiments. 1-Butanol was added at three concentrations: 0.1 mM, 2 mM and 40 mM. Cells were incubated in 1-butanol for 5, 10, 30, 60 and 120 min. The total RNA was extracted and blotted onto Nytran membranes (Schleicher and Schuell, Keene, NH, U.S.A.) for analysis by northern hybridization. RNA from lactate-grown cells was used as a negative control while RNA from cells exposed to butane for 2 h were used as a positive control. The same membranes, then, were stripped and re-hybridized with

a 16S ribosomal RNA (16S rRNA) probe. The probes for *boh*, *bdh* and 16S rRNA were prepared as previously described (Vangnai *et al.*, 2002).

4.3.4 Determination of 1-butanol oxidation by gas chromatography

The concentration of substrate utilized was determined in a Shimadzu GC-8A gas chromatograph (Shimadzu Corporation, Tokyo, Japan) equipped with a flame-ionization detector and 60 cm long by 0.1 cm inside-diameter stainless steel column packed with Porapak Q (Waters, Milford, Mass.). The column temperature was 160 °C.

4.3.5 Measurements of whole cell respiration activities and inhibition of the respiration

The *P. butanovora* wild-type cells, *boh::tet* mutant cells and *bdh::kan* mutant cells were grown on 2 mM 1-butanol to late exponential phase (35-40 h). Then, cells were harvested, washed twice with 25 mM phosphate buffer pH 7.0 to remove the remaining substrate. Cells were resuspended and concentrated in 25 mM phosphate buffer pH 7.0 and then kept at room temperature (~25°C) for at least 1 h to lower the endogenous respiration. The whole-cell O₂ consumption was measured at 30°C using a Clark style Oxygen electrode (Yellow Springs Instruments Co.) with a 2-ml reaction volume. The chamber was filled with 25 mM phosphate buffer pH 7.0, to which 40-100 µl of cell suspension was added, followed by the addition of substrate, 1-butanol, to the final concentration of 2 mM. Inhibition of respiration was determined following addition of inhibitors from stock solution. Potassium cyanide (Sigma) (1 mM, 100 mM and 200 mM) was prepared in 25 mM phosphate buffer pH 7.0. Salicylhydroxamic acid (500 mM and 1 M),

n-propyl gallate (500 mM and 1M), and antimycin A (500 mM) (Sigma, St. Louis, Mo.) were dissolved in dimethylsulfoxide (DMSO). When 1 % DMSO (vol/vol) was added into the reaction, it decreased the O₂ consumption rate by 2%. Therefore, to avoid the solvent effect, less than 1 % (vol/vol) final volume of each inhibitor solubilized in DMSO was used. The values reported were corrected for endogenous respiration (typically less than 15% of the rate obtained when 1-butanol was added) and for the presence of solvent, if necessary. Lines were fitted to the data sets using a curve-fitting software program (SigmaPlot 4.0, Jandel Scientific, Corte Madera., U.S.A.). The inhibitor concentration required for half-maximal inhibition (IC₅₀) and the percent maximal inhibition were reported. Inhibition experiments were repeated 4-9 times with similar results. The values reported are means of at least 4 independent preparations.

4.4 RESULTS

4.4.1 Comparison of kinetic constants for BOH and BDH

To gain insight into the role of BOH in 1-butanol metabolism, we determined some of the kinetic constants and compared these to previously determined values for BDH. Partially purified BOH was used for these determinations. BOH has high preference towards 1-butanol with a K_m of 1.7 ± 0.2 μM . This result shows that 1-butanol, the product of butane oxidation in the terminal oxidation pathway, is a good substrate for both BOH and BDH (K_m for 1-butanol = 7 ± 1 μM) (Vangnai & Arp, 2001). Butyraldehyde is also a substrate for BOH. However, the K_m of BOH towards butyraldehyde was 369 ± 85 μM , which indicates a strong preference for 1-butanol. A similar situation was observed for

BDH with a K_m for butyraldehyde of $535 \pm 13 \mu\text{M}$ (Vangnai & Arp, 2001). Unlike BDH, BOH has a broader substrate range which includes the ability to oxidize 2-butanol (Vangnai *et al.*, 2002). In contrast to the preference for 1-butanol as the substrate, BOH has a much lower preference for 2-butanol with a K_m of $662 \pm 98 \mu\text{M}$. For BOH, the rates obtained at saturating concentration of 2-butanol and butyraldehyde were 72% and 66%, respectively, of that obtained with 1-butanol. For type I quinoprotein ADH from *P. aeruginosa* (ATCC 17933), the K_m 's for 2-butanol and *S*(+)-2-butanol were $680 \mu\text{M}$ and $980 \mu\text{M}$, respectively, while the K_m 's for the primary alcohols ethanol and 1-propanol were $14 \mu\text{M}$ and $21 \mu\text{M}$, respectively (Rupp & Gorisch, 1988). In *P. putida* HK5, the K_m 's for ethanol and 1-butanol were $163 \mu\text{M}$ and 1.62 mM , respectively (Toyama *et al.*, 1995).

4.4.2 The induction of *boh* mRNA and *bdh* mRNA in *P. butanovora* in response to different levels of 1-butanol

Although both BOH and BDH share similar affinities towards 1-butanol, we considered the possibility that BOH and BDH were expressed differently in response to 1-butanol. Using northern hybridization, the inductions of *boh* mRNA and *bdh* mRNA in response to different levels of 1-butanol were tested. Lactate-grown wild-type *P. butanovora* cells were harvested, washed, resuspended in carbon source-free medium and divided into five parts. One part was incubated with lactate added to the medium. One part was exposed to butane (10% vol/vol gas phase). The three remaining parts were then exposed to either a non-toxic level (0.1 mM), a growth-supporting level (2 mM) or a toxic level (40 mM) of 1-butanol. Then all parts were incubated with shaking for 3 h. A 16S rRNA probe confirmed that equivalent mRNA amounts were loaded into the gel for each treatment (data not shown). Multiple bands observed in some cases may be caused by the

degradation of *boh* mRNA and *bdh* mRNA, as there was no *boh* mRNA or *bdh* mRNA induced in cells incubated with lactate. *P. butanovora* responded quickly when exposed to 1-butanol (Fig. 4.1) as *boh* mRNA and *bdh* mRNA were detectable within the first 5 min of exposure at all levels of 1-butanol and continued to increase for at least 60 min. Some differences between the expression patterns for *boh* and *bdh* were observed, i.e. a maximum of *boh* mRNA induction was observed at 60 min of exposure while *bdh* mRNA was still increasing after 60 min. However, the patterns for each gene were not markedly different over the three 1-butanol concentrations tested. Overall, we did not observe strong differences in the expression levels of BOH and BDH in response to different levels of 1-butanol. This conclusion is consistent with the similar kinetic constant for each enzyme.

4.4.3 The toxicity effect of 1-butanol and butyraldehyde towards cell growth

Next, we considered the possibility that BOH and BDH served different roles in the detoxification of 1-butanol. To determine the efficiency of each enzyme in the detoxification of 1-butanol, growth of the wild-type, a mutant lacking BOH (the *boh::tet* strain), and a mutant lacking BDH (the *bdh::kan* strain) were compared in media containing 2 mM citrate supplemented with different levels of 1-butanol (0 mM, 2 mM, 10 mM, 40 mM, 60 mM and 80 mM). Citrate was chosen because it does not repress the expression of *boh* and *bdh*. Wild-type cells could tolerate higher levels of 1-butanol than either of the single mutants (Fig. 4.2A, B, and C). 1-Butanol (up to 40 mM) enhanced growth of wild-type cells (Fig. 4.2A). In contrast, 1-butanol became toxic to the mutant strains at concentrations greater than 10 mM. For example, at 40 mM 1-butanol the *boh::tet* strain and the *bdh::kan* strain grew more slowly than wild-type cells and reached

optical densities that were 35% and 50%, respectively, of that of wild-type cells (Fig. 4.2B and C).

This result was consistent with our previous observation that when cells with either *boh* or *bdh* disrupted were grown in 2 mM 1-butanol (without citrate), the cultures reached final optical densities that were half those observed in wild-type cells (Vangnai *et al.*, 2002). 1-Butanol consumption by cells grown on 2 mM citrate and supplemented with 2 mM 1-butanol at the stationary phase was determined. Wild-type *P. butanovora*, the *boh::tet* strain, and the *bdh::kan* strain showed 1-butanol consumption activities of 123 ± 5 , 53 ± 5 , and 62 ± 3 nmole \cdot min⁻¹ \cdot mg protein⁻¹, respectively. Overall, these results indicate that cells with both BOH and BDH can tolerate higher levels of 1-butanol and can benefit to a greater extent from the presence of 1-butanol. Again, no substantial differences were noted in the ability of either 1-butanol dehydrogenase to respond to 1-butanol toxicity.

The oxidation of 1-butanol yields butyraldehyde. Cells can metabolize butyraldehyde to butyrate, which then probably enters the β -oxidation pathway for fatty acids. Butyraldehyde is also toxic to cells. Because BOH and BDH are able to oxidize butyraldehyde, we determined how well cells respond to different levels of butyraldehyde if either BOH or BDH was lacking. Growth of wild-type cells and mutant cells in low concentrations of butyraldehyde (2 mM and 4 mM) was significantly slowed due to the high toxicity of butyraldehyde (Fig. 4.3). However, there was no significant difference of growth rates among wild-type cells and both mutants grown in citrate-containing medium supplemented with butyraldehyde. This result suggested that BOH and BDH are less important in terms of butyraldehyde oxidation and detoxification in *P. butanovora*.

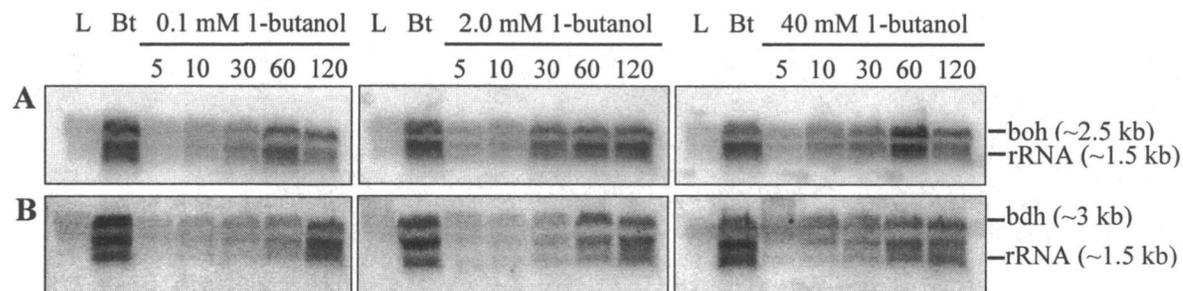
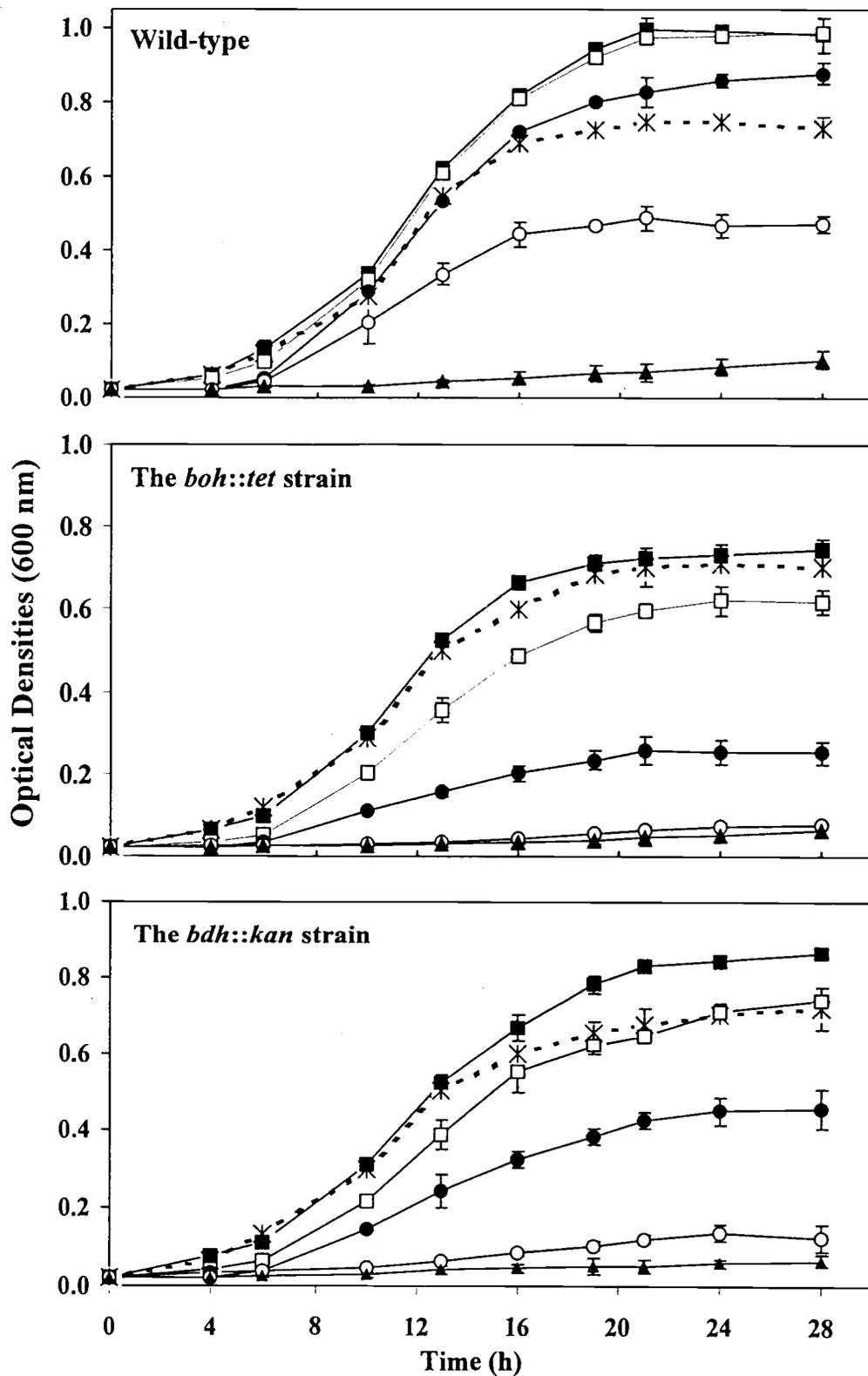


Figure 4.1 The induction of *boh* mRNA and *bdh* mRNA at different levels of 1-butanol. Wild-type *P. butanovora* were grown on 4 mM lactate overnight, then cells were harvested for the induction experiments. Cells were exposed to 1-butanol at different levels: 0.1 mM (a non-toxic level), 2 mM (a growth-supporting level) and 40 mM (a toxic level for the mutants) for 5, 10, 30, 60 and 120 minutes. Northern hybridization was performed with (A) a *boh* probe, or (B) a *bdh* probe. L and Bt represent RNA from lactate-grown cells and RNA from butane-induced cells used as a negative control and a positive control for the induction, respectively.

Figure 4.2 The effect of 1-butanol on the growth of *P. butanovora* wild-type, *boh::tet* and *bdh::kan* strains on citrate. Wild-type *P. butanovora*, the *boh::tet* strain, and the *bdh::kan* strain were grown on 2-mM citrate-containing medium supplemented with 0, 2, 10, 40, 60 or 80 mM 1-butanol. Cell growth was measured and compared to growth of cells in citrate-containing medium.

.....*..... citrate grown cells, —■— citrate +2 mM 1-butanol,
—□— citrate +10 mM 1-butanol, —●— citrate +40 mM 1-butanol,
—○— citrate + 60 mM 1-butanol, and —▲— citrate +80 mM 1-butanol.

Figure 4.2



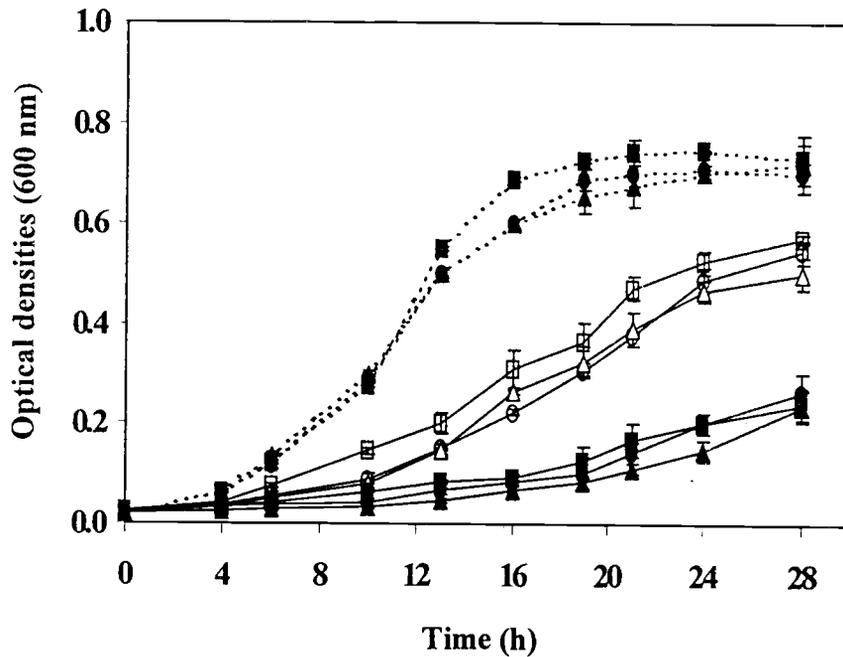


Figure 4.3 The effect of butyraldehyde on the growth of *P. butanovora* wild type, *boh::tet* and *bdh::kan* strains on citrate. Cells were grown on 2-mM citrate-containing medium supplemented with 0, 2 or 4 mM butyraldehyde. In order, growth of wild-type *P. butanovora*, the *boh::tet* strain and the *bdh::kan* strain:■.....,●.....,▲..... citrate-grown cells
 —□—, —○—, —△— citrate-grown cells + 2 mM butyraldehyde
 —■—, —●—, —▲— citrate-grown cells + 4 mM butyraldehyde

4.4.4 Two distinct terminal oxidase systems in *P. butanovora* in response to 1-butanol oxidation and detoxification

We investigated the possibility that BOH and BDH served different bioenergetic roles in the metabolism of 1-butanol. Different respiratory inhibitors were used to investigate putative routes of electrons from 1-butanol oxidation through the respiratory chain and to the terminal oxidase complex(s) (including the alternative oxidase).

Wild-type *P. butanovora*, the *boh::tet* strain, and the *bdh::kan* strain were grown in 2 mM 1-butanol to stationary phase (35-40 h), then 1-butanol-dependent O₂ consumption and 1-butanol consumption were determined. 1-Butanol-dependent O₂ consumption rates for wild-type cells, the *boh::tet* strain, and the *bdh::kan* strain were 126 ± 21 , 60 ± 11 , and 70 ± 7 nmole O₂ consumed • min⁻¹ • mg protein⁻¹, respectively, and their 1-butanol consumption activities were 135 ± 16 , 75 ± 11 , and 81 ± 4 nmole 1-butanol oxidized • min⁻¹ • mg protein⁻¹, respectively. These activities were similar to the 1-butanol consumption activities we obtained for cells grown in citrate supplemented with 2 mM 1-butanol. These results suggested that 1-butanol was not completely oxidized to CO₂ under the assay conditions.

Antimycin A was used as an inhibitor of the cytochrome *b-c₁* region of complex III (ubiquinol: cytochrome *c* oxidoreductase) (Singer, 1979). The inhibition patterns of cells treated with different concentrations of antimycin A varied markedly in response to which butanol dehydrogenases were present (Fig. 4.4). 1-Butanol-dependent O₂ uptake by BOH in the *bdh::kan* strain was strongly inhibited by low concentrations of antimycin A (IC₅₀ of 0.33 mM; Table 4.1). On the other hand, the inhibition of 1-butanol-dependent O₂ uptake of wild-type cells and the *boh::tet* strain by antimycin A was not significant (Fig. 4.4). The antimycin A inhibition patterns suggested that electrons from BOH follow a different pathway of electron transport from that involving electrons from BDH.

To further test this possibility, cyanide was used as an inhibitor of electron transport. In wild-type cells, 1-butanol dependent O₂ uptake exhibited a biphasic inhibition pattern when treated with cyanide (Fig. 4.5) indicating that there are two inhibition sites (half-maximal inhibition occurs at ~0.10 mM and ~0.20 mM). Electron transport coupled to BOH as revealed in the *bdh::kan* strain was sensitive to cyanide (IC₅₀ of 0.09 mM) and was inhibited completely (96 ± 5 %) when treated with 1 mM cyanide (Fig. 4.5 and Table 4.1). Electron transport coupled to BDH as revealed in the *boh::tet* strain, on the other hand, was less sensitive to cyanide (IC₅₀ of 0.20 mM) and only 62 ± 10 % inhibition was achieved when treated with 1 mM cyanide.

Cyanide inhibition of butyraldehyde-dependent O₂ uptake was also determined. Complete inhibitions (90%-97%) were observed in all cell types (data not shown). Electron transport coupled to BOH was more sensitive to cyanide (IC₅₀ of 0.06 mM) than electron transport coupled to BDH (IC₅₀ of 0.20 mM). Although BOH and BDH exhibited different sensitivities by cyanide inhibition in response to butyraldehyde oxidation, no biphasic inhibition pattern was observed in wild-type *P. butanovora* (IC₅₀ of 0.08).

Table 4.1 Inhibition of 1-butanol-dependent whole cell respiration of wild-type *P. butanovora*, the *boh::tet* and the *bdh::kan* strains, grown on 1-butanol. Respiration was measured in the presence of 2 mM 1-butanol as substrate. IC₅₀ is the inhibitor concentration required for half-maximal (or achieved) inhibition.

Enzyme present	Wild-type <i>P. butanovora</i>		The <i>boh::tet</i> strain		The <i>bdh::kan</i> strain	
	BOH and BDH		BDH		BOH	
Inhibitor (tested concentrations)	IC ₅₀ (mM)	Maximal inhibition (%)	IC ₅₀ (mM)	Maximal inhibition (%)	IC ₅₀ (mM)	Maximal inhibition (%)
Antimycin A (0-1 mM)	-	15 ± 3*	-	6 ± 4*	0.33	58 ± 4
SHAM (0-3 mM)	1.09	58 ± 7*	1.01	79 ± 11*	1.58	30 ± 6*
<i>n</i> -Propyl gallate (0-3 mM)	0.77	78 ± 13	0.66	91 ± 7	1.38	42 ± 5
KCN (0-2 mM)	0.10 [†] , 0.20 [†]	89 ± 14	0.20	62 ± 10	0.09	96 ± 5

* Achieved inhibition obtained in the range of inhibitor used.

[†] The value was estimated from the biphasic inhibition curve (Fig 4.5).

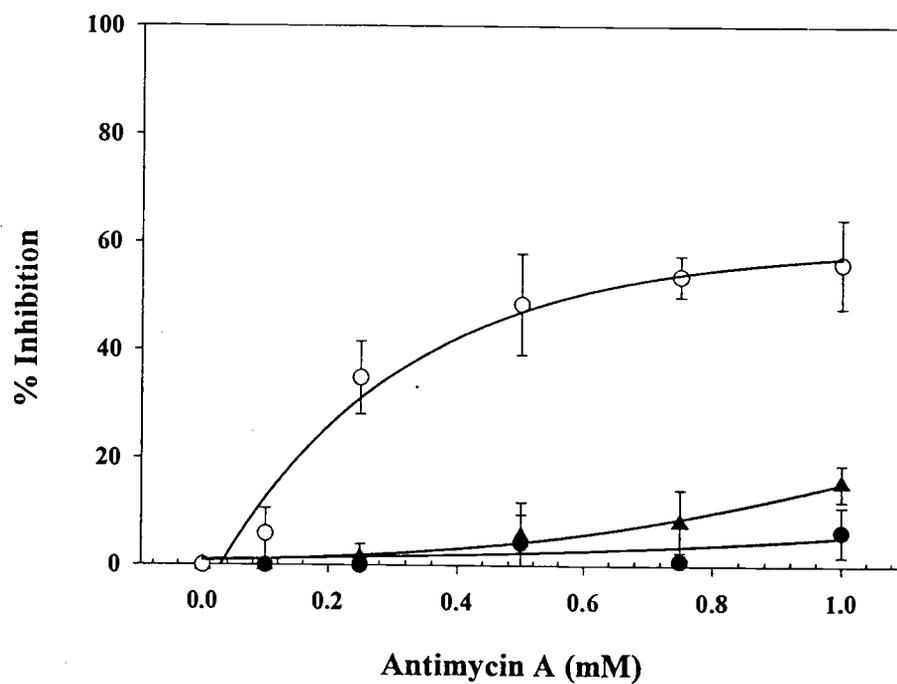


Figure 4.4 Inhibition of 1-butanol-dependent whole cell respiration by antimycin A. 1-Butanol grown cells were treated with antimycin A (0-1 mM). —▲— Wild-type *P. butanovora*, —●— the *boh::tet* strain (carrying BDH), and —○— the *bdh::kan* strain (carrying BOH). The results were from 4 independent replicates.

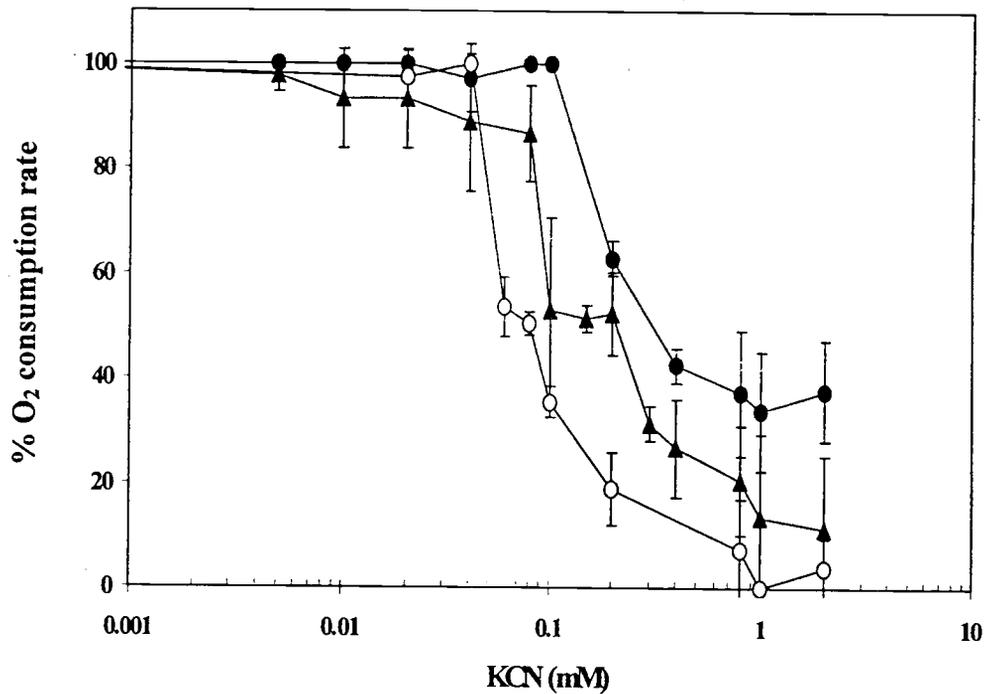


Figure 4.5 Residual 1-butanol-dependent whole cell respiration following treatment with potassium cyanide. 1-Butanol grown cells were treated with potassium cyanide (0-2 mM). —▲— Wild-type *P. butanovora*, —●— the *boh::tet* strain (carrying BDH), and —○— the *bdh::kan* strain (carrying BOH). The results were from 9 independent replicates.

Electron transport was further tested with salicylhydroxamic acid (SHAM), an inhibitor of the alternative terminal oxidase of plant mitochondria that is less sensitive to cyanide (Schonbaum *et al.*, 1971). The concentration of SHAM required for half-achieved inhibition (IC_{50}) of 1-butanol-dependent O_2 uptake by the *boh::tet* strain (carrying BDH) was 1.01 mM (Fig. 4.6 and Table 4.1). Moreover, 1-butanol-dependent respiration of the *bdh::kan* strain was only inhibited $30 \pm 6\%$ by 3 mM SHAM compared to $79 \pm 11\%$ in the *boh::tet* strain. When the *boh::tet* strain was treated with 2 mM cyanide and 3 mM SHAM, the 1-butanol-dependent O_2 uptake was completely inhibited ($97 \pm 4\%$). Besides SHAM, *n*-propyl gallate, a potent inhibitor of the alternative, cyanide-less-sensitive pathway (Siedow & Girvin, 1980) was used to confirm the presence of the alternative oxidase system in 1-butanol grown *P. butanovora*. The strong inhibition of the 1-butanol-dependent O_2 uptake in the *boh::tet* strain when treated with *n*-propyl gallate (up to 3 mM) confirmed the result obtained with SHAM (Table 4.1). The effect of respiratory inhibitors shown in Fig. 4.5, Fig. 4.6 and Table 4.1 suggested that 1-butanol-dependent respiratory pathway used by BDH (in the *boh::tet* strain) is the alternative, cyanide-less-sensitive system.

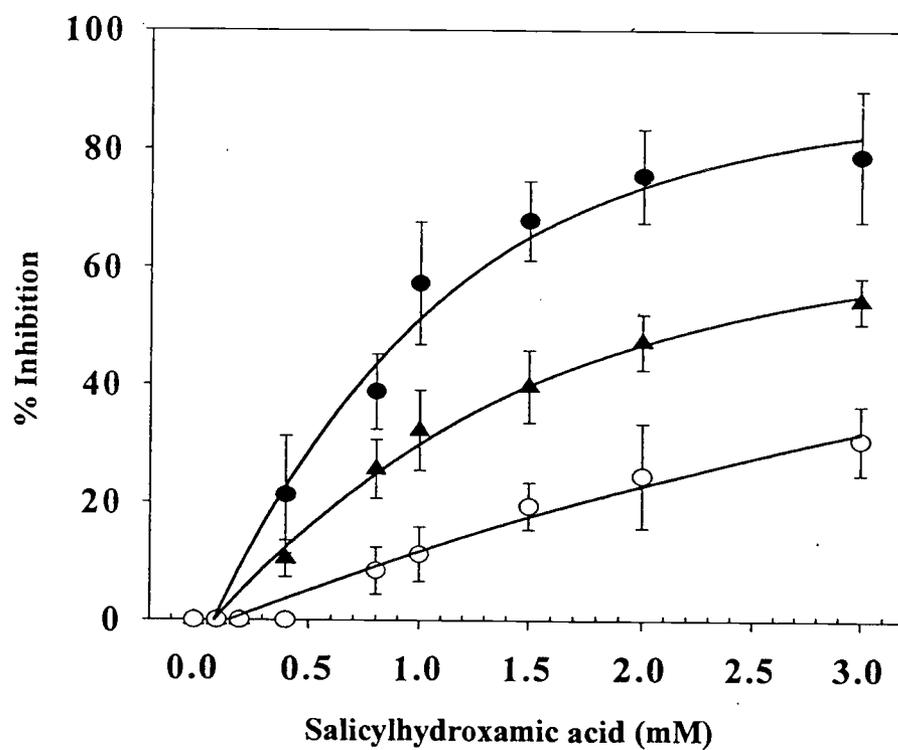


Figure 4.6 Inhibition of 1-butanol-dependent whole cell respiration by salicylhydroxamic acid (SHAM). 1-Butanol grown cells were treated with SHAM (0-3 mM). —▲— Wild-type *P. butanovora*, —●— the *boh::tet* strain (carrying BDH), and —○— the *bdh::kan* strain (carrying BOH). The results were from 4 independent replicates.

4.5 DISCUSSION

Pseudomonas butanovora grown on butane or 1-butanol expresses two 1-butanol dehydrogenases, a quinoprotein BOH and a quinohemoprotein BDH. The presence of multiple alcohol dehydrogenases in one organism is not without precedent. In most cases different ADHs were expressed when cells were induced with different substrates or multiple ADHs expressed simultaneously had different substrate ranges. In contrast, in *P. butanovora*, BOH and BDH were induced with and oxidize the same substrate, 1-butanol. In this study, we investigated the similarities and dissimilarities of BOH and BDH regarding 1) their kinetic characteristics, 2) the expression patterns of the genes coding for each enzyme, 3) their roles in 1-butanol detoxification and 4) their physiological function, i.e. bioenergetic role. BOH and BDH each have high preference towards 1-butanol as the substrate (2 and 7 μM , respectively) and fair preference towards butyraldehyde (535 and 369 μM , respectively). BOH can oxidize 2-butanol, although the K_m is comparatively high. Thus, both BOH and BDH seem to function primarily in the oxidation of 1-butanol, the product of butane metabolism through the terminal oxidation pathway. Moreover, *boh* mRNA and *bdh* mRNA exhibit clear induction when exposed to 1-butanol over a wide range of 1-butanol concentrations. Thus, the principal role of each 1-butanol dehydrogenase is not to respond differently to a particular 1-butanol concentration.

Although 1-butanol is a growth substrate for *P. butanovora*, higher concentrations inhibit growth. The availability of mutants with either butanol dehydrogenase missing coupled with the fact that cells express the butanol dehydrogenases when citrate is available for growth, gave us a system to examine independently the role of each enzyme in protecting cells from 1-butanol toxicity. Wild-type cells contained twice as much 1-butanol consumption activity compared

to that of each single mutant and were able to tolerate higher levels of 1-butanol. On the contrary, mutant cells were sensitive to lower concentration of 1-butanol and reached lower cell densities. The results suggested that both enzymes play a role in allowing cells to tolerate higher concentrations of 1-butanol.

We further investigated the physiological function of BOH and BDH in the context of the electron transport chains in *P. butanovora*. Inhibition of O₂ uptake by antimycin A, an inhibitor of Complex III, leads us to conclude that at least some of the electrons released in the metabolism of 1-butanol must pass through ubiquinone when only BOH is present. Surprisingly, when BDH is present, either alone or with BOH, electrons apparently can bypass Complex III. Antimycin A could not completely inhibit the electron transport chain when BOH is present (only 58 ± 4 % maximal inhibition) suggesting that even in this case a portion of the electron flow could bypass Complex III. Our results with cyanide and SHAM are consistent with the presence of two distinct terminal oxidase systems in *P. butanovora* with different coupling to each butanol dehydrogenase. As shown above, the 1-butanol-dependent O₂ uptake that occurs when only BOH is present is highly sensitive to cyanide, while that of cells containing only BDH is less sensitive to cyanide. Interpretation of the effects of respiratory inhibitors in whole cells is complicated by the fact that 1-butanol oxidation yields butyraldehyde which is then oxidized by at least two NAD⁺-dependent butyraldehyde dehydrogenases. The electrons from butyraldehyde oxidation are expected to pass through Complex III and to couple to the cyanide-sensitive pathway. For example, the partial inhibition caused by cyanide when only BOH was present may be due to the inhibition of butyraldehyde metabolism, while the residual activity may be due to electron flow from BDH to the cyanide less-sensitive terminal oxidase.

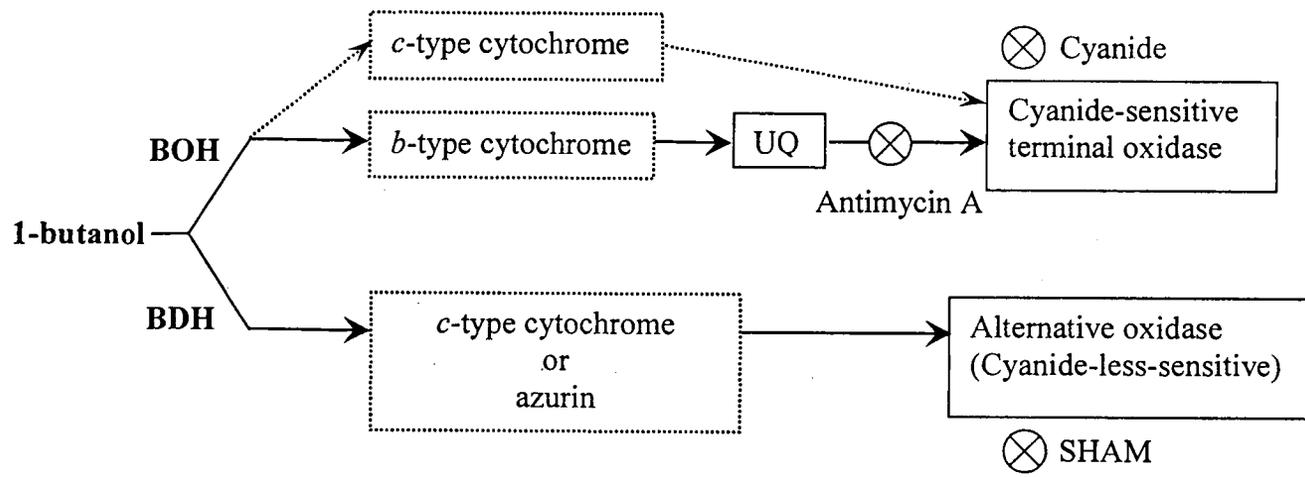
In *Pseudomonas* sp. (Matsushita *et al.*, 1982) and *Acinetobacter calcoaceticus* (Beardmore-Gray & Anthony, 1986), ubiquinone was found to be an electron acceptor of membrane-associated quinoprotein glucose dehydrogenase.

Glucose is oxidized in the periplasm and the electrons are then transported to the membrane-spanning regions in the N-terminal region of glucose dehydrogenase. Ubiquinone was also recently suggested to serve as an electron acceptor in periplasmic PQQ-containing ADH in *P. putida* HK5 (Matsushita *et al.*, 2000) and in soluble glucose dehydrogenase through cytochrome *b₅₆₂* (Anthony, 1993; Docker *et al.*, 1986; Hauge, 1961). In other type I quinoproteins, cytochrome *c_{EDH}* (Schrover *et al.*, 1993) and cytochrome *c₅₅₀* (Schobert & Gorisch, 1999) were reported to serve as electron acceptors. BOH appears to function like the ADH in *P. putida* HK5. In contrast, BDH apparently does not transfer electrons to ubiquinone, rather, electrons seem to flow to an alternative oxidase. The presence of cyanide-less-sensitive system associated with BDH was confirmed by the inhibition of 1-butanol dependent O₂ uptake by SHAM and *n*-propyl gallate. SHAM was found to be an inhibitor of the NADH-dependent cyanide-less-sensitive pathway reported in *P. aeruginosa* strain PAO6049 (Cunningham & Williams, 1995), while it had no effect on the cyanide-less-sensitive oxidase system in *P. aeruginosa* IFO 3445 (Matsushita *et al.*, 1983). High concentrations of hydroxamic acids used to inhibit the alternative oxidase pathway had no discernible effect on either the respiratory pathway through cytochrome oxidase, or on the energy coupling reactions (Schonbaum *et al.*, 1971). In other periplasmic quinoxinoproteins (type II), the *c*-type cytochrome or a blue copper protein were suggested to function as the electron acceptor (Anthony, 1998; Goodwin & Anthony, 1998). A blue copper protein, azurin, was suggested to be an electron-transfer mediator in ADH IIB of *P. putida* HK5 (Matsushita *et al.*, 1999; Matsushita *et al.*, 2000). Recently, the alcohol oxidation activity through ADH IIB in *P. putida* HK5 was also proposed to have two different electron transport systems, a cyanide-sensitive oxidase and a cyanide-less-sensitive oxidase in the intact cells (Matsushita *et al.*, 2000).

We propose a schematic model for 1-butanol-dependent respiration in *P. butanovora* (Fig. 4.7). Since 1-butanol-dependent O₂ uptake initiated by BOH is coupled to ubiquinone, then to the cyanide-sensitive terminal oxidase, this pathway is expected to contribute to the generation of the proton motive force. Cyanide-less-sensitive and SHAM-sensitive alternative oxidases typically are not capable of contributing to the proton motive force. If this were true in *P. butanovora* as well, then the 1-butanol oxidation-dependent electron transport chain that utilizes BDH would not couple to an energy-generating respiratory complex. Instead, the electrons are transferred to an unknown electron acceptor(s), then to cyanide-less-sensitive pathway. An uncoupled pathway for 1-butanol oxidation would appear to provide an ideal mechanism to detoxify 1-butanol, assuming that cells could rapidly remove the even more toxic product, butyraldehyde. On the other hand, a coupled pathway would appear to provide cells with an advantage when using 1-butanol as a growth substrate. When 1-butanol is oxidized by either BOH or BDH in *P. butanovora*, butyraldehyde is produced. Butyraldehyde is further oxidized by aldehyde dehydrogenase(s) to butyrate, which then probably enters the β -oxidation pathway. We have identified, in *P. butanovora*, two putative aldehyde dehydrogenase genes, which are in close proximity to the genes coding for BOH and BDH (Vangnai *et al.*, 2002). The similarity of their sequences to known aldehyde dehydrogenases suggests that they are NAD⁺-dependent enzymes. This result suggests that aldehyde oxidation (through these two enzymes) is coupled with the Complex I (NADH dehydrogenase) of the respiratory chain and, therefore, the oxidation of butyraldehyde would provide more energy for cell growth than does 1-butanol oxidation.

Further elucidation of the electron transport chains associated with each butanol dehydrogenase will require investigations with reconstituted vesicles where the complications of butyraldehyde metabolism can be mitigated.

Figure 4.7 Schematic model for 1-butanol-dependent respiratory systems in *P. butanovora*.



4.6 ACKNOWLEDGMENTS

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CHAPTER 5.

CONCLUSIONS

5.1 SUMMARY

The oxidation of gaseous *n*-alkanes initiated by monooxygenase(s) in microorganisms was studied because of the versatile substrate specificities and the potential use in the bioremediation of recalcitrant compounds (Burback & Perry, 1993; Hamamura *et al.*, 1997; Vanderburg *et al.*, 1995).

The metabolisms of butane, a representative of short-chain gaseous *n*-alkane, have been most extensively described in *Corynebacterium-Nocardia-Mycobacterium-Rhodococcus* complex of Gram positive bacterium. One notable exception is the butane metabolism demonstrated in a Gram negative bacterium, *Pseudomonas butanovora*. In *P. butanovora*, butane is oxidized by a soluble butane monooxygenase (Arp lab, unpublished) at the terminal carbon yielding 1-butanol as the predominant product (Arp, 1999; Hamamura *et al.*, 1999). Up to 90% of the butane consumed was accounted for as 1-butanol (in the presence of 1-propanol as an inhibitor for 1-butanol oxidation) (Arp, 1999) confirming the terminal pathway for the butane oxidation. Although the subterminal oxidation product, 2-butanol, was not detected, it supported cell growth and it could be consumed by butane-grown cells (Arp, 1999). To further investigate the pathway for the oxidation of 1-butanol and 2-butanol induced in butane-grown *P. butanovora*, I focused on the characterization of alcohol dehydrogenase(s) involved in the butane metabolic pathway.

In Chapter 2, a 1-butanol dehydrogenase (BDH) induced in butane-grown *P. butanovora* was purified to near homogeneity and identified as an NAD⁺-independent, type II quinoxinoprotein ADH. It was shown to be primarily

responsible for 1-butanol oxidation in the butane metabolic pathway. BDH is the first quinohemoprotein ADH reported to associate with alkane oxidation by bacteria (Toyama *et al.*, 1995; Vangnai & Arp, 2001). BDH exhibits its highest activity and preference towards 1-butanol. A broad range of compounds, including primary alcohols, secondary alcohols, aldehydes, C₄-diols, and aromatic alcohols are substrates. However, 2-butanol can not be oxidized by BDH. An NAD⁺-dependent secondary ADH was observed in butane-grown *P. butanovora*, which may suggest a low level of the subterminal butane oxidation pathway (Arp, 1999).

The gene encoding BDH was further examined (Chapter 3). The *bdh* gene shows high similarity (70% - 72%) with other type II quinohemoproteins in ethanol-grown *C. testosteroni* and tetrahydrofurfuryl alcohol-grown *R. eutropha*. Another 1-butanol dehydrogenase (BOH) gene in *P. butanovora* was found in close proximity to the gene coding for aldehyde dehydrogenase. The deduced amino acid sequence of BOH suggests a type I quinoprotein ADH and has high similarity (80%) to type I quinoprotein ethanol dehydrogenases from *P. aeruginosa*. The involvement of BOH and BDH in butane metabolism in *P. butanovora* was confirmed. In the presence of butane, both *boh* and *bdh* mRNAs were induced along with the 1-butanol oxidation activity. The *P. butanovora* mutant with both *boh* and *bdh* genes disrupted was unable to grow on butane or 1-butanol. To further examine the biochemical and kinetic characteristics of BOH, BOH was partially purified from cell extracts of *P. butanovora* strain with the *bdh* gene inactivated. BOH exhibits relatively high activity with 1-butanol, but has a broader substrate range than that of BDH including an ability to oxidize a wider range of secondary alcohols such as 2-propanol and 2-butanol. 2-Butanol also induces *boh* mRNA expression, but not *bdh* mRNA expression.

Because both BOH and BDH oxidize 1-butanol most efficiently, the role(s) of each enzyme in the dual pathway for 1-butanol oxidation in *P. butanovora* was questioned. Therefore, I further investigated the similarity and dissimilarity of the

role(s) of each 1-butanol dehydrogenase in butane and 1-butanol metabolism in Chapter 4. Interestingly, the results presented in Chapter 4 suggest that BOH and BDH do not respond differently to 1-butanol because both enzymes have high activities and the similar low range of K_m towards 1-butanol (2 and 7 μM , respectively). Furthermore, the mRNA induction profiles of BOH and BDH confirmed that *boh* and *bdh* mRNAs were induced similarly over the three different 1-butanol concentrations tested. The unique roles of BOH and BDH became clearer when I measured the growth of wild-type cells, the *boh::tet* strain, and the *bdh::kan* strain in the citrate-containing growth medium supplemented with different levels of 1-butanol. The wild-type *P. butanovora* could tolerate higher levels of 1-butanol than the mutants. This result suggested the detoxification role of one of the enzymes. I continued the investigation with the possibility that BOH and BDH serve different physiological functions in the metabolism of 1-butanol. From the studies described in Chapter 4, two distinct terminal oxidase systems in *P. butanovora* in response to 1-butanol oxidation and detoxification were discovered. The electrons transported from 1-butanol oxidation appeared to follow a branched electron transport chain. BOH may be coupled to ubiquinone with the electrons being transported to a cyanide-sensitive terminal oxidase suggesting a function primarily in energy-generation. In contrast, the electrons from BDH may be linked with a cyanide-less-sensitive terminal oxidase, which suggests a role of BDH primarily in the detoxification of 1-butanol.

5.2 CONCLUDING STATEMENTS

The metabolism of gaseous *n*-alkanes in bacteria is poorly understood. The research presented in this thesis is another interesting step to our understanding of the metabolic pathway of butane, a representative for short-chain gaseous *n*-alkane,

through a dual pathway of 1-butanol oxidation. A quinoprotein (BOH) and a quinohemoprotein (BDH) relating to the *n*-alkane and primary alcohol oxidation were discovered and characterized biochemically, genetically, and physiologically in *P. butanovora*. The observation of two distinct terminal oxidase systems in alkane/alcohol-oxidizing bacteria is the beginning of studies that will lead us to understand the pathway of butane metabolism.

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