Roles for the Two 1-Butanol Dehydrogenases of *Pseudomonas butanovora* in Butane and 1-Butanol Metabolism

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*Pseudomonas butanovora* grown on butane or 1-butanol expresses two 1-butanol dehydrogenases, a quinoprotein (BOH) and a quinohemoprotein (BDH). BOH exhibited high affinity towards 1-butanol ($K_m = 1.7 \pm 0.2$ $\mu$M). BOH also oxidized butyraldehyde and 2-butanol ($K_m = 369 \pm 85$ $\mu$M and $K_m = 662 \pm 98$ $\mu$M, respectively). The mRNA induction profiles of BOH and BDH at three different levels of 1-butanol, a nontoxic level (0.1 mM), a growth-supporting level (2 mM), and a toxic level (40 mM), were similar. When cells were grown in citrate-containing medium in the presence of different levels of 1-butanol, wild-type *P. butanovora* could tolerate higher levels of 1-butanol than the *P. butanovora boh::tet* strain and the *P. butanovora bdh::kan* strain. A model is proposed in which 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.

*Pseudomonas butanovora* ATCC 43655 is a gram-negative, rod-shaped bacterium that was isolated from activated sludge from an oil-refining plant by using *n*-butane as the energy source (36). *P. butanovora* can utilize a variety of organic compounds as growth substrates, including $C_2$ to $C_6$ $n$-alkanes, the corresponding primary alcohols, carboxylic acids, and some polyvalent alcohols, but not alkenes, sugars, or $C_1$ compounds (35, 36). Butane-grown *P. butanovora* can degrade some chlorinated aliphatic hydrocarbons (18) and thus has potential for bioremediation of sites contaminated with these solvents. The pathway of butane metabolism in butane-grown *P. butanovora* was determined to follow the terminal oxidation pathway, that is, butane $\rightarrow$ 1-butanol $\rightarrow$ butyraldehyde $\rightarrow$ butyrate (6).

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* PNKb1, *Rhodococcus rhodochrous* NRRL B-1244 were purified and characterized as multiple ADHs in alkane-utilizing and alcohol-utilizing bacteria (37). Type III ADHs are membrane-associated enzymes found in the cytoplasmic membrane of *M. vaccae* (37). Type I ADH uses a c-type cytochrome or copper-containing protein (azurin) (4, 26). Type II ADHs are soluble periplasmic quinohemoproteins having PQO and heme c as prosthetic groups and have been found in *Comamonas testosteroni* (12, 17), *P. putida* (37), and *Ralstonia eutropha* (40). When *P. putida* HK5 is grown on ethanol, 1-butanol, and 1,2-propanediol, it produces three different quinoprotein ADHs: one type I ADH and two type II ADHs (ADH IIb and ADH IIg), respectively (37). Type III ADHs are membrane-associated enzymes found in the cytoplasmic membrane of acetate acid bacteria. Type III ADHs have three subunits: a quinohemoprotein, a trimeric cytochrome $c$, and a subunit of unknown function (1, 22). The electron acceptor of type III ADHs is ubiquinone (4, 23).

In butane-grown *P. butanovora*, five different ADHs with different specificities towards primary and secondary alcohols were identified (38, 39). Among these, *P. butanovora* expresses two distinct NAD$^+$-independent POQ-containing 1-butanol dehydrogenases, BOH (a quinoprotein) and BDH (a quino-
moprotein). The substrate range of BOH and its gene were characterized previously (39). BOH is a 64-kDa type I quinoprotein without its putative 29-residue leader sequence and is located in the periplasm. BDH has also been characterized biochemically and genetically (38, 39). BDH is a soluble, periplasmic, type II quinohemoprotein that contains 1.0 mol of POQ and 0.25 mol of heme c as prosthetic groups and exists as a monomer with an apparent molecular mass of 67 kDa (38). 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 rates of both mutant strains on butane were decreased, but eventually the organisms reached optical densities similar to that observed for wild-type cells. Growth of the mutant strains on 1-butanol resulted in final densities that were one-half that observed for 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 the essential role of these proteins in the butane and 1-butanol oxidation pathway (39). However, the previous studies did not reveal 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 of these enzymes 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 in this paper. 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 below.

MATERIALS AND METHODS

Cell culture and chemicals. Cells of P. butanovora were grown in sealed serum bottles (150 ml) as previously described (35) 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 ensure an adequate supply of O2 to the cells. For growth with 1-butanol, the substrate was added into the medium at a final concentration of 2 mM. For growth with 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 turbidities of 600 nm of about 0.5 were observed.

Wild-type P. butanovora ATCC 43655 was obtained from the American Type Culture Collection. The boh::tet strain has the boh gene inactivated by insertion of a tetracycline resistance (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 insertion of a transposon containing a kanamycin resistance (kan) gene 715 nucleotides downstream of the ATG start codon of bdh (39).

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

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 mutant strain bdh::kan. Cells of the bdh::kan strain grown on 2 to 4 mM 1-butanol were harvested at an optical density at 600 nm of 0.5, washed twice, and resuspended at a concentration of 1.5 g/ml in 25 mM MOPS (morpholinopropanesulfonic acid) buffer (pH 7.0). The cell suspension was frozen at −20°C for at least 24 h and then thawed at room temperature. For cell lysis preparation, lysozyme (0.2 mg/ml), a small amount of DNA nuclease I, and MgSO4 (final concentration, 2 mM) were added to the cell suspension. The mixture was gently homogenized with a precooled glass homogenizer and then gently rocked at room temperature for 1 h. Unbroken cells were removed by 15 min of centrifugation at 11,000 × g at 4°C. The membrane fraction was separated by centrifugation at 200,000 × g (SW40 rotor, Beckman L8-70 centrifuge) for 1 h at 4°C. The supernatant containing BOH was kept at −80°C until it was used.

BOH was partially purified by the following steps. In each step, all fractions or pool of each step, preincubated with POQ on ice for 1 h, were examined for 1-butanol-dependent phenazine methosulfate (PMS) reductase activity, and this was followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. The PMS reductase activity was measured spectrophotometrically as described previously (38), 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 the next column.

(i) First Q-Sepharose FPLC. The cell supernatant of the bdh::kan mutant was applied to a Q-Sepharose (Sigma) anion-exchange fast protein liquid chromatography (FPLC) column (2.2 by 18 cm; Millipore Corp.) which had been equilibrated with 25 mM MOPS buffer (pH 7.0). The proteins were eluted with a linear 0 to 1 M NaCl gradient in the same buffer. BOH eluted at 190 to 300 mM NaCl.

(ii) Superose 6 FPLC. The active Q-Sepharose fractions were pooled, dialyzed, and then applied to a Superose 6 (Sigma) gel filtration column (1.1 by 80 cm).

(iii) Second Q-Sepharose FPLC. Active fractions from the Superose 6 column were pooled and concentrated with a centrifugal filter membrane (Centricron YM30; Amicon, Millipore Corp.), which removed molecules smaller than 30 kDa, before being applied to a Q-Sepharose column (2.2 by 10 cm). The proteins were eluted with a continuous-step gradient consisting of 0 to 80 mM NaCl (1 column volume [CV]), 80 to 350 mM NaCl (1 CV), 350 to 400 mM NaCl (1 CV), and 400 mM to 1 M NaCl. Active fractions from the second Q-Sepharose column, which eluted at 80 to 350 mM NaCl, were then pooled and concentrated with a centrifugal filter membrane (Centricron YM50; Amicon, Millipore Corp.), which removed molecules smaller than 50 kDa. The partially purified BOH produced two predominant bands, at approximately 63 and 64 kDa, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The partially purified BOH, which exhibited a sixfold increase in specific activity, was used to determine the Km of the enzyme. The Km's were calculated from the initial rates determined by the PMS reductase standard assay previously described (38) by varying the concentrations of substrates tested from 0.1 μM to 1 mM. The protein concentration was fixed at 60 nM.

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 in induction buffer (1 liter) and divided for induction experiments. 1-Butanol was added at three concentrations: 0.1, 2, and 40 mM. Cells were incubated with 1-butanol for 5, 10, 30, 60, and 120 min. The total RNA was extracted and blotted onto Nytran membranes (Schleicher and Schuell, Keene, N.H.) 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 was used as a positive control. The same membranes were then stripped and rehybridized with a 16S rRNA probe. The probes for boh, bdh, and 16S rRNA were prepared as previously described (39).

Determination of 1-butanol oxidation by gas chromatography. The concentration of substrate utilized was determined with a Shimadzu GC-8A gas chromatograph (Shimadzu Corporation, Tokyo, Japan) equipped with a flame ionization detector and a stainless steel column (length, 60 cm; inside diameter, 0.1 cm) packed with Porapak Q (Waters, Milford, Mass.). The column temperature was 160°C.

Measurements of whole-cell respiration activities and inhibition of respiration. P. butanovora wild-type cells, boh::tet mutant cells, and bdh::kan mutant cells were grown on 2 mM 1-butanol to the late exponential phase (35 to 40 h). Then cells were harvested and washed twice with 25 mM phosphate buffer (pH 7.0) to remove the remaining substrate. Cells were resuspended, 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 by using a Clark style oxygen electrode (Yellow Springs Instrument Co.) with a 2-ml reaction volume. The chamber was filled with 25 mM phosphate buffer (pH 7.0), to which 40 to 100 μl of cell suspension was added, and then substrate (1-butanol) was added to a final concentration of 2 mM. Inhibition of respiration was determined following addition of inhibitors from stock solutions. Potassium cyanide (1, 100, and 200 mM; Sigma) was prepared in 25 mM phosphate buffer (pH 7.0). Salicylhydroxamic acid (SHAM) (500 mM and 1 M), n-propyl gallate (500 mM and 1 M), and antimycin A (500 mM; Sigma) were dissolved in dimethyl sulfoxide (DMSO). When 1% (vol/vol) DMSO was added to the reaction mixture, it decreased the O2 consumption rate by 2%. Therefore, to avoid a solvent effect, the final volume of each inhibitor solution added to the DMSO used (with 1% vol/vol of the total volume) of respiratory inhibitors did not substantially inhibit BOH and BDH activities. The activities of partially purified BOH and purified BDH were inhibited 3 and 12%, respectively, by antimycin A (1 mM) and 5 and 7%, respectively, by SHAM (3 mM). No inhibition of the 1-butanol oxidation activity of either enzyme was...
detected in the presence of cyanide (1 mM). The values reported here 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 by using an exponential curve-fitting software program (SigmaPlot 4.0; Jandel Scientific, Corte Madera, Calif.). The inhibitor concentrations required for half-maximal inhibition (IC50) and the percentages of maximal inhibition are reported below. Inhibition experiments were repeated four to nine times with similar results. The values reported below are means for at least four independent preparations.

RESULTS

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 them to previously determined values for BDH. Partially purified BOH was used for these determinations. The specific activity of the partially purified BOH towards 1-butanol was 0.82 ± 0.13 μmol · min⁻¹ · mg of protein⁻¹, while that of the purified BDH was 4.82 ± 0.34 μmol · min⁻¹ · mg of protein⁻¹ (38). BOH has high affinity for 1-butanol, with a Kₘ of 1.7 ± 0.2 μM. This result shows that both BOH and BDH (Kₘ for 1-butanol, 7 ± 1 μM) (38) express high affinity for 1-butanol, the product of butane oxidation in the terminal oxidation pathway. Butyraldehyde is also a substrate for BOH. However, the Kₘ of BOH for butyraldehyde was 369 ± 85 μM, which indicates a strong preference for 1-butanol. A similar situation was observed for BDH, which had a Kₘ for butyraldehyde of 535 ± 13 μM (38). Compared with BDH, BOH has a broader substrate range, which includes the ability to oxidize 2-butanol (39). In contrast to the ability for 1-butanol, BOH has a much lower affinity for 2-butanol, with a Kₘ of 662 ± 98 μM. For BOH, the rates obtained with saturating concentrations of 2-butanol and butyraldehyde were 72 and 66%, respectively, of the rate obtained with 1-butanol. For type I quinoprotein ADH from P. aeruginosa ATCC 17933, the Kₘ for 2-butanol and S(±)-2-butanol were 680 and 980 μM, respectively, while the Kₘ for the primary alcohols ethanol and 1-propanol were 14 and 21 μM, respectively (28). For P. putida HK5, the Kₘ₇ for ethanol and 1-butanol were 163 μM and 1.62 mM, respectively (37).

Induction of boh mRNA and bdh mRNA in P. butanovora in response to different levels of 1-butanol. Although BOH and BDH have similar affinities for 1-butanol, we considered the possibility that BOH and BDH were expressed differently in response to 1-butanol. By using Northern hybridization, the induction of boh mRNA and the induction of 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-free basal medium, and divided into five parts. One part was incubated with lactate added to the medium, and one part was exposed to butane (10% [vol/vol] in the gas phase). The three remaining parts were then exposed to either a nontoxic 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 all treatments (data not shown). Multiple bands observed in some cases may have been caused by degradation of boh mRNA and bdh mRNA as there was no boh mRNA or bdh mRNA induced in cells incubated in lactate-containing medium. Southern hybridizations also showed that the probes are specific for single genes (39), eliminating the possibility of other induced isoenzymes. P. butanovora responded quickly when it was exposed to 1-butanol (Fig. 1), as boh mRNA and bdh mRNA were detectable within the first 5 min of exposure at all levels of 1-butanol and the levels continued to increase for at least 60 min. Some differences between the expression patterns for boh and bdh were observed; i.e., the maximum level of boh mRNA induction was observed after 60 min of exposure, while the level of bdh mRNA was still increasing after 60 min. However, the patterns for each gene were not markedly different for 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.

Toxicity effect of 1-butanol and butyraldehyde towards cell growth. Next, we considered the possibility that BOH and BDH had 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, growth of the mutant lacking BOH (the boh::kan strain), and growth of the mutant lacking BDH (the bdh::kan strain) were compared in media containing 2 mM citrate supplemented with different levels of 1-butanol (0, 2, 10, 40, 60, 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. 2). 1-Butanol at concentrations up to 40 mM enhanced the growth of wild-type cells (Fig. 2A). In contrast, 1-butanol inhibited the growth of the mutant strains at concentrations greater than 10 mM. For example, in the pres-
ence of 40 mM 1-butanol the growth rates of the boh::tet strain (0.23 h⁻¹) and the bdh::kan strain (0.26 h⁻¹) were less than the growth rate of the wild type (0.36 h⁻¹), and the final optical densities were 35 and 50%, respectively, of the final optical density of the wild-type cells (Fig. 2B and C).

These results were consistent with our previous observation that when cells with either boh or bdh disrupted were grown with 2 mM 1-butanol as the only carbon source, the cultures reached final optical densities that were one-half those observed for wild-type cells (39). 1-Butanol consumption by cells grown on 2 mM citrate and 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 nmol · min⁻¹ · mg of 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 responded to different levels of butyraldehyde if either BOH or BDH was lacking. Growth of wild-type cells and mutant cells in the presence of low concentrations of butyraldehyde (2 and 4 mM) was significantly slowed due to the high toxicity of butyraldehyde (Fig. 3). However, there was no significant difference in the growth rates of wild-type cells and the two 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.

Two distinct terminal oxidase systems in P. butanovora in response to 1-butanol oxidation and detoxification. We investigated the possibility that BOH and BDH have 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 to the terminal oxidase complex(es) (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 the stationary phase (35 to 40 h), and then 1-butanol-dependent O₂ consumption and 1-butanol consumption rates were determined. The 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 nmol of O₂ consumed · min⁻¹ · mg of protein⁻¹, respectively, and the 1-butanol consumption activities were 135 ± 16, 75 ± 11, and 81 ± 4 nmol of 1-butanol oxidized · min⁻¹ · mg of protein⁻¹, respectively. These activities were
similar to the 1-butanol consumption activities which 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) (34). The inhibition patterns of cells treated with different concentrations of antimycin A varied markedly in response to which butanol dehydrogenases were present (Fig. 4). 1-Butanol-dependent O₂ uptake by BOH in the boh::kan strain was strongly inhibited by low concentrations of antimycin A (IC₅₀, 0.33 mM) (Table 1). On the other hand, the inhibition of 1-butanol-dependent O₂ uptake in wild-type cells and the boh::tet strain by antimycin A was not significant (Fig. 4). The antimycin A inhibition patterns suggested that electrons from BOH follow a different pathway of electron transport than from electrons from BDH follow.

To further test this possibility, cyanide was used as an inhibitor of electron transport. In wild-type cells, 1-butanol-dependent O₂ uptake by the boh::tet strain was sensitive to cyanide (IC₅₀, 0.08 mM). Although BOH and BDH exhibited different sensitivities to cyanide inhibition in response to butyraldehyde oxidation, no biphasic inhibition pattern was observed in wild-type P. butanovora (IC₅₀, 0.08 mM).

Electron transport was further tested with SHAM, an inhibitor of the alternative terminal oxidase of plant mitochondria that is less sensitive to cyanide (30). The IC₅₀ of SHAM for 1-butanol-dependent O₂ uptake by the boh::tet strain was 1.01 mM (Fig. 6 and Table 1). Moreover, 1-butanol-dependent respiration of the boh::kan strain was inhibited only 30% ± 6% by 3 mM SHAM, compared to 79% ± 11% for the

![Image](http://jb.asm.org/)

**FIG. 4.** Inhibition of 1-butanol-dependent whole-cell respiration by antimycin A. 1-Butanol-grown cells were treated with antimycin A (0 to 1 mM). Symbols: ▲, wild-type P. butanovora; ●, boh::tet strain (carrying BDH); ○, boh::kan strain (carrying BOH). The results were obtained from four independent replicates.

![Image](http://jb.asm.org/)

**FIG. 5.** Residual 1-butanol-dependent whole-cell respiration following treatment with potassium cyanide. 1-Butanol-grown cells were treated with potassium cyanide (0 to 2 mM). Symbols: ▲, wild-type P. butanovora; ●, boh::tet strain (carrying BDH); ○, boh::kan strain (carrying BOH). The results were obtained from nine independent replicates.

<table>
<thead>
<tr>
<th>Inhibitor (tested concn)</th>
<th>Wild-type P. butanovora (with BOH and BDH)</th>
<th>boh::tet strain (with BDH)</th>
<th>boh::kan strain (with BOH)</th>
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<tr>
<td></td>
<td>IC₅₀ (mM)</td>
<td>Maximal inhibition (%)</td>
<td>IC₅₀ (mM)</td>
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<tr>
<td>Antimycin A (0–1 mM)</td>
<td>1.38 ± 0.16</td>
<td>42 ± 2%</td>
<td>0.33</td>
</tr>
<tr>
<td>SHAM (0–3 mM)</td>
<td>1.09</td>
<td>58 ± 7%</td>
<td>1.01</td>
</tr>
<tr>
<td>α-Propyl gallate (0–3 mM)</td>
<td>0.77</td>
<td>78 ± 13%</td>
<td>0.66</td>
</tr>
<tr>
<td>KCN (0–2 mM)</td>
<td>ND*</td>
<td>89 ± 14%</td>
<td>0.20</td>
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</table>

* Respiration was measured in the presence of 2 mM 1-butanol as the substrate.

* Achieved inhibition obtained with the range of inhibitor concentrations used.

* Not determined.
cyanide. fication, and (iv) the physiological functions of the genes coding for each enzyme, (iii) the roles of the enzymes in the characteristics of the enzymes, (ii) the expression patterns of the alternative oxidase system in 1-butanol-grown P. butanovora. Strong inhibition of 1-butanol-dependent O2 uptake in the boh::tet strain when it was treated with n-propyl gallate (at concentrations up to 3 mM) confirmed the results obtained with SHAM (Table 1). The effects of respiratory inhibitors, shown in Fig. 5 and 6 and Table 1, suggested that the 1-butanol-dependent respiratory pathway used by BDH (in the boh::tet strain) is the alternative system that is less sensitive to cyanide.

**DISCUSSION**

*P. butanovora* grown on butane or 1-butanol expresses two 1-butanol dehydrogenases, the quinoprotein BOH and the quinohemoprotein BDH. The presence of multiple ADHs in one organism is not without precedent. In most cases different ADHs are expressed when cells are induced with different substrates, or multiple ADHs expressed simultaneously have different substrate ranges. In contrast, in *P. butanovora*, BOH and BDH were induced with and oxidized the same substrate, 1-butanol. In this study, we investigated the similarities and dissimilarities of BOH and BDH with respect to (i) the kinetic characteristics of the enzymes, (ii) the expression patterns of the genes coding for each enzyme, (iii) the roles of the enzymes in 1-butanol detoxification, and (iv) the physiological functions (i.e., bioenergetic roles) of the enzymes. Both BOH and BDH have high affinity for 1-butanol (2 and 7 μM, respectively) and fair affinity for butyraldehyde (535 and 369 μM, respectively). BOH can oxidize 2-butanol, although the affinity is comparatively low. Thus, both BOH and BDH seem to function primarily in the oxidation of 1-butanol, the product of butane metabolism in the terminal oxidation pathway. Moreover, boh mRNA and bdh mRNA exhibit clear induction when cells are exposed to 1-butanol at a wide range of 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 one of the butanol dehydrogenases 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 exhibited twice as much 1-butanol consumption activity as each single mutant and were able to tolerate higher levels of 1-butanol. In contrast, mutant cells were sensitive to lower concentrations of 1-butanol and reached lower cell densities. Our results suggested that both enzymes play a role in allowing cells to tolerate higher concentrations of 1-butanol. In bacteria, in addition to the physiochemical changes in the membrane when cells cope with solvent stress, two energy-dependent biochemical mechanisms involved in the reduction of toxic organic solvents have been observed: (i) a solvent exclusion system and (ii) metabolic removal via oxidation (20, 27). Our studies focused on the latter system in *P. butanovora*.

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, led us to conclude that at least some of the electrons released during 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 BDH was present (the maximal inhibition was only 58% ± 4%), suggesting that even in this case a portion of the electron flow could bypass complex III. Our results obtained with cyanide and SHAM are consistent with the presence of two distinct terminal oxidase systems in *P. butanovora* with differences in 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 the 1-butanol-dependent O2 uptake 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 only when BOH was present may have been due to the inhibition of butyraldehyde metabolism, while the residual activity may have been due to electron flow from BDH to the terminal oxidase less sensitive to cyanide.

In *Pseudomonas* sp. (21) and *Acinetobacter calcoaceticus* (9), ubiquinone was found to be an electron acceptor of the 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 (26) and in solu-
FIG. 7. Schematic model for 1-butanol-dependent respiratory systems in P. butanovora. The inhibition sites of antimycin A, cyanide, and SHAM are each indicated by a multiplication sign in a circle. UQ, ubiquinone.

ble glucose dehydrogenase through cytochrome $b_{552}$ (5, 13, 19). In other type I quinoproteins, cytochrome $c_{550}$ (31) and cytochrome $c_{550}$ (29) have been 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 a system less sensitive to cyanide that is associated with BDH was confirmed by the inhibition of 1-butanol-dependent $O_2$ uptake by SHAM and n-propyl gal late. SHAM was found to be an inhibitor of the NADH-dependent pathway less sensitive to cyanide reported in $P$. aeruginosa strain PAO6049 (11), while it had no effect on the oxidase system less sensitive to cyanide in $P$. aeruginosa IFO 3445 (24). 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 the energy-coupling reactions (30). In other periplasmic quinohemoproteins (type II), the $c$-type cytochrome or a blue copper protein was suggested to function as the electron acceptor (4, 14). A blue copper protein, azurin, was suggested to be an electron transfer mediator in ADH IIB of $P$. putida HK5 (25, 26). 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 an oxidase less sensitive to cyanide in the intact cells (26).

We propose a schematic model for 1-butanol-dependent respiration in $P$. butanovora (Fig. 7). Since 1-butanol-dependent $O_2$ uptake initiated by BOH is coupled to ubiquinone and then to the cyanide-sensitive terminal oxidase, this pathway is expected to contribute to generation of the proton motive force. Less cyanide-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) and then to a pathway less sensitive to cyanide. An uncoupled pathway for 1-butanol oxidation appears to provide an ideal mechanism to detoxify 1-butanol, assuming that cells can rapidly remove the product that is even more toxic, butyraldehyde. On the other hand, a coupled pathway appears to provide cells with an advantage when 1-butanol is used 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 an aldehyde dehydrogenase(s) to butyrate, which then probably enters the $\beta$-oxidation pathway. We have identified in $P$. butanovora two putative aldehyde dehydrogenase genes, which are close to the genes coding for BOH and BDH (39). The similarity of the sequences of these enzymes to the sequences of known aldehyde dehydrogenases suggests that they are NAD$^+$-dependent enzymes. This result suggests that aldehyde oxidation (through these two enzymes) is coupled with complex I (NADH dehydrogenase) of the respiratory chain, and therefore, the oxidation of butyraldehyde should provide more energy for cell growth than 1-butanol oxidation provides.

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

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