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Evidence for Involvement of Copper Ions and Redox State in Regulation of Butane Monooxygenase in *Pseudomonas butanovora*▽

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Pseudomonas butanovora **possesses an alcohol-inducible alkane monooxygenase, butane monooxygenase (BMO), that initiates growth on C_2-C_9 alkanes. A *lacZ* transcriptional reporter strain, *P. butanovora bmoX*::*lacZ*, in which the BMO promoter controls the expression of β-galactosidase activity, was used to show that 1-butanol induced the BMO promoter in the presence or absence of O_2 when lactate-grown, BMO-repressed cells were washed free of lactate and incubated in NH_4Cl-KNa phosphate buffer. In contrast, when lactate-grown cells of the reporter strain were incubated in phosphate buffer containing the mineral salts of standard growth medium, 1-butanol-dependent induction was significantly repressed at low O_2 (1 to 2% [vol/vol]) and totally repressed under anoxic conditions. The repressive effect of the mineral salts was traced to its copper content. In cells exposed to 1% (vol/vol) O_2, $CuSO_4$ (0.5 μM) repressed 1-butanol-dependent induction of β-galactosidase activity. Under oxic conditions (20% O_2 [vol/vol]), significantly higher concentrations of $CuSO_4$ (2 μM) were required for almost complete repression of induction in lactate-grown cells. A combination of the Cu^{2+} reducing agent Na ascorbate (100 μM) and $CuSO_4$ (0.5 μM) repressed the induction of β-galactosidase activity under oxic conditions to the same extent that 0.5 μM $CuSO_4$ alone repressed it under anoxic conditions. Under oxic conditions, 2 μM $CuSO_4$ repressed induction of the BMO promoter less effectively in butyrate-grown cells of the *bmoX*::*lacZ* strain and of an R8-*bmoX*::*lacZ* mutant reporter strain with a putative BMO regulator, BmoR, inactivated. Under anoxic conditions, $CuSO_4$ repression remained highly effective, regardless of the growth substrate, in both BmoR-positive and -negative reporter strains.**

A model has been developed for the regulation of butane monooxygenase (BMO) in the C_2-C_9 alkane-utilizing bacterium *Pseudomonas butanovora* (6–8, 23, 24). The salient features of this model are as follows. (i) BMO transcription is up-regulated by alcohol, aldehyde, and epoxide products of alkane and alkene oxidation (7, 8, 24). (ii) Recently, a gene (*bmoR*) upstream of the *bmo* operon that is involved in induction of BMO by alcohols of chain length C_2 to C_8 was identified (15). (iii) BMO transcription is down-regulated in response to the presence of the organic acid propionate, a downstream product of odd-chain-length alkane oxidation (8). (iv) Both even- and odd-chain-length organic-acid products of alkane oxidation, e.g., propionate and butyrate, are potent in vivo inhibitors and inactivators of the BMO enzyme in the absence of alkane substrates (6). The net result is the close coupling of alkane oxidation to the cells' demands for C and energy and the ability of the cell to consume the products of alkane oxidation.

Under anoxic conditions, *P. butanovora* can utilize various alcohols, aldehydes, and organic acids for growth using nitrate as a terminal electron acceptor (D. M. Doughty, D. J. Arp, and P. J. Bottomley, unpublished data). Because the activity of the BMO enzyme is dependent upon O_2 as a cosubstrate for catalytic activity, the induction of BMO in response to alcohols and aldehydes under anoxic conditions would be superfluous. Our studies were extended to determine if alcohol induction of BMO was overridden in the absence of O_2. During the course of the work, 1-butanol-dependent induction of BMO transcription was found to be unaffected when aerobically grown cells were suspended in buffer and transitioned into low-O_2 conditions. In contrast, when cells were resuspended in the complete mineral salts of standard growth medium under low-O_2 conditions, BMO induction was repressed. Because of the novelty of this observation, the objectives of this study were (i) to identify the component(s) of the mineral salts causing the repressive effect, (ii) to determine if the effect was redox influenced, and (iii) to eliminate the possibility that the repression was due to metal toxicity. Subsequently, copper ions were identified as the causative agent of the repressive effect. Because copper ions are involved in repression of the soluble form of methane monooxygenase (sMMO) in methanotrophs (4, 11, 19, 22), and since sMMO is structurally and biochemically similar to BMO (3, 13, 26), our findings are discussed in the context of a recently published model that links the role of copper ions in sMMO expression with the redox state of a regulatory protein, MmoS (28).

MATERIALS AND METHODS

Bacterial strains, growth conditions, and routine assays. *P. butanovora* (ATCC 43655) is a gram-negative bacterium that grows on *n*-alkanes C_2 to C_9 (8, 27) and is closely related to the betaproteobacterial genera *Azoarcus* and *Thauera* (1). Liquid cultures were grown as previously described (7), with NH_4Cl (2 mM) supplied as an N source and KNO_3 (30 mM) added where indicated to serve as the electron acceptor. For anoxic growth, the medium was autoclaved, degassed by aspiration, flushed with N_2 for 30 min to remove residual O_2, and autoclaved a second time for sterilization. When organic acids or alcohols served as C sources for growth, concentrations were balanced to 12 mM carbon equiv-

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alents, sufficient to support the growth of *P. butanovora* to an optical density at 600 nm (OD_{600}) of 0.6 (0.3 mg protein · ml^{-1}).

Determination of induction and repression of β-galactosidase expression in *lacZ* transcriptional reporter strains of *P. butanovora*. The *P. butanovora bmoX*::*lacZ*::*kan* strain contains a bicistronic expression system in which kanamycin resistance is constitutive and the BMO promoter controls the expression of β-galactosidase activity (24). *bmoR*, a gene encoding a putative transcriptional regulator, was studied by mutational analysis and was determined to be involved in the induction of the BMO promoter (15). A BmoR-deficient variant of the *bmoX*::*lacZ*::*kan* strain (an R8-*bmoX*::*lacZ* strain) was constructed (15). Although β-galactosidase activity was not induced by alcohols in either the acetate or lactate-grown R8-*bmoX*::*lacZ* strain, when the R8-*bmoX*::*lacZ* strain was grown on C_3, C_4, or C_5 fatty acids to repress BMO expression, β-galactosidase was induced by C_2-C_5 alcohols. These data imply that a second BMO regulatory system exists in *P. butanovora*, albeit one that is expressed only during growth on propionate, butyrate, or valerate. Experiments were carried out to determine to what extent BmoR-dependent and -independent induction of the BMO promoter was sensitive to $CuSO_4$. Both of the *lacZ* reporter strains (*bmoX*::*lacZ* and R8-*bmoX*::*lacZ* strains) were grown on organic acids or alcohols under the same conditions described for the wild-type cells. Induction assays were performed as described previously (5), with the modification that the cells were resuspended either in 50 mM KNa phosphate-2 mM NH_4Cl, pH 7.1; in 50 mM KNa phosphate-2 mM NH_4Cl, pH 7.1, plus the complete mineral salts of standard growth medium; or in 50 mM KNa phosphate-2 mM NH_4Cl, pH 7.1, plus individual trace metal salts of the growth medium. Preliminary experiments were conducted to ensure that the trace metal salts tested for BMO repression were not inhibitory to β-galactosidase activity at the concentrations tested. For experiments carried out under subambient O_2 concentrations, O_2 was flushed from 10-ml vials with N_2 (30 ml/s for 30 s). Anoxic growth medium or buffer was prepared as described above, and aliquots (1 ml) were transferred with an airtight syringe to induction vials. Anoxic solutions of the individual trace element compounds used in the growth medium ($MnCl_2 \cdot 4H_2O$, $FeSO_4 \cdot 7H_2O$, $CoSO_4 \cdot 7H_2O$, $NiCl_2 \cdot 6H_2O$, $CuSO_4 \cdot 5H_2O$, and $ZnSO_4 \cdot 7H_2O$) and others not used in the growth medium ($CdCl_2$, $HgCl_2$, AgCl, and $PtCl_2$) were prepared as 100 μM stock solutions and made anoxic by flushing them with N_2. The salt solutions were transferred into assay vials using an airtight syringe. Specific amounts of O_2 were added to each vial, and the values are expressed as percent O_2 (volume per total volume of headspace in the vial). A high concentration (approximately 10 mg protein · ml^{-1}) of washed cells was resuspended in 50 mM KNa phosphate buffer, pH 7.1, and was made anoxic by flushing them with N_2. Aliquots of the concentrated cell suspension (5 μl) were injected into the induction vials to a final OD_{600} of 0.2 (0.1 mg protein · ml^{-1}). Additional replicates of low-O_2 and anoxic treatments were set up to confirm the presence or absence of O_2 at the beginning and end of each experiment by the methylene blue oxidation assay (18). A series of experiments were conducted to assess whether the effect of $CuSO_4$ under oxic conditions could be enhanced by the addition of the Cu^{2+} reducing agent ascorbic acid (20, 30). Stock solutions of 10 mM Na ascorbate were prepared daily in KNa phosphate buffer and used at a final concentration of 100 μM.

Induction of propionate consumption in lactate-grown *P. butanovora*. The effects of $CuSO_4$ and O_2 on the induction of propionate metabolism were examined. Cells grown under oxic conditions in the presence of lactate were harvested, washed three times, resuspended in NH_4Cl-KNa phosphate buffer, and made anoxic by flushing them with N_2. The cells were injected into 160-ml vials containing 50 ml of anoxic NH_4Cl-KNa phosphate buffer. O_2 (1% [vol/total volume of headspace]) was added to the headspace of the vials, and $CuSO_4$ (0.5 μM) and rifampin (40 μg · ml^{-1}) were added as indicated. Propionate consumption was induced by the addition of propionate (2 mM, final concentration). To confirm that propionate metabolism was not induced in the absence of propionate, control vials that did not receive propionate were included. Following 2 h of incubation, the cells were harvested and washed three times in phosphate buffer, and propionate consumption was determined as described previously (8, 13).

Determination of the effect of the $CuSO_4$ concentration on O_2 consumption in lactate-grown *P. butanovora*. Endogenous and 1-butanol-dependent O_2 uptake were followed using a Clark style O_2 electrode in a glass reaction vial (YSI model 5300 Biological Oxygen Monitor; Yellow Springs Co., Yellow Springs, OH) as described previously (7). Cells were grown aerobically on lactate, harvested, and resuspended in NH_4Cl-KNa phosphate buffer containing either 0, 0.1, 0.3, 1, 3, 10, or 30 μM $CuSO_4$. After 2 h, the cells were washed, resuspended in buffer without $CuSO_4$, and assayed for lactate-dependent O_2 consumption.

RESULTS

Repression of β-galactosidase in the *bmoX*::*lacZ* reporter strain of *P. butanovora* during growth on 1-butanol under nitrate-respiring conditions. Preliminary data were obtained showing that *P. butanovora* grown under oxic conditions on 1-butanol as the sole C source expressed BMO activity approximately 20% that of butane-grown cells (data not shown). In contrast, BMO activity was not detected in cells grown under anoxic conditions on 1-butanol as the C source and with KNO_3 as an electron acceptor (data not shown). Because these data do not distinguish an effect of anoxic growth on transcription of the *bmo* operon from conditions unfavorable for the development of functional BMO per se, a transcriptional *bmoX*::*lacZ* reporter strain was used to study the effect of anoxia on transcription of the *bmo* operon. β-Galactosidase activity was detected at high levels in the *bmoX*::*lacZ* reporter strain during oxic growth on 1-butanol and was not significantly affected by the presence or absence of nitrate (Fig. 1A and B). In contrast, β-galactosidase activity was much lower during growth on 1-butanol under anoxic conditions with KNO_3 supplied as the electron acceptor (Fig. 1C). These data show that 1-butanol induction of *bmo* transcription is repressed during growth of *P. butanovora* under anoxic denitrifying conditions and that KNO_3 does not repress β-galactosidase under oxic conditions.

Effect of percent O_2 on 1-butanol-dependent β-galactosidase expression in the *bmoX*::*lacZ* reporter strain. The sensitivity of 1-butanol-dependent induction of β-galactosidase to O_2 levels was explored. When cells of the lactate-grown *bmoX*::*lacZ* reporter strain were resuspended in NH_4Cl/KNa phosphate buffer with or without KNO_3, the level of 1-butanol-dependent induction of β-galactosidase activity did not change significantly as O_2 decreased from 20% to 0%; the induction of the BMO promoter was reduced somewhat by the presence of KNO_3 at ≥2% O_2 (Fig. 2A and B). When cells were resuspended in the phosphate-buffered complete mineral salts of standard growth medium, there was a striking reduction in the expression of β-galactosidase activity in the absence of O_2 regardless of the presence or absence of KNO_3 (Fig. 2C and D). These data suggest that transcriptional repression of the *bmo* promoter under anoxic conditions requires a component of the growth medium and that low O_2 concentrations per se, with or without the presence of KNO_3, were insufficient to cause transcriptional repression.

Effect of transition metals on 1-butanol-dependent induction of BMO at 20% and 1% O_2. Preliminary experiments pointed to the trace element mixture of the growth medium being responsible for the repression effect. We examined the effects of individual transition metal components of the growth medium on 1-butanol-dependent induction of β-galactosidase activity at low (1%) and ambient (20%) O_2 levels (Table 1). Although $CuSO_4$ (0.5 μM) had no effect on 1-butanol-dependent induction of β-galactosidase at ambient O_2, induction was reduced to ≤25% of the buffer control at 1% O_2. However, $CuSO_4$ exerted a repressive effect of similar magnitude under oxic conditions when its concentration was raised to 2 μM (Fig. 3A). Other transition metals included in the growth medium did not significantly enhance or diminish the ability of 1-butanol to induce BMO transcription under either O_2 level. An analysis of other transition metals that are not components of

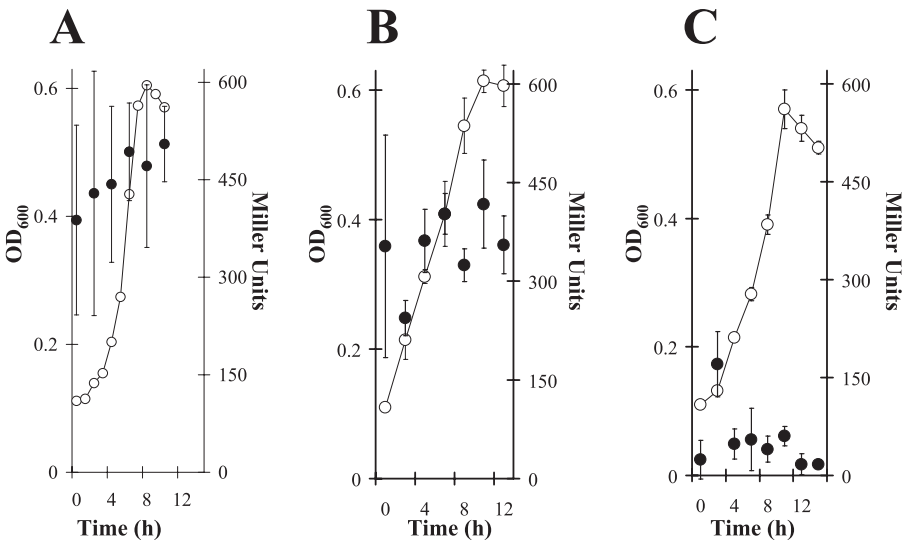

FIG. 1. The effects of O_2 and KNO_3 on expression of β-galactosidase activity in the *bmoX*::*lacZ* reporter strain of *P. butanovora* during growth on 1-butanol. The open symbols represent growth measured by OD_{600}, and the closed symbols represent β-galactosidase activity expressed in Miller units. (A) *bmoX*::*lacZ* reporter strain grown under oxic conditions on 1-butanol (3 mM) in standard growth medium. (B) *bmoX*::*lacZ* reporter strain grown under oxic conditions on 1-butanol (3 mM) with KNO_3 (30 mM) added to the growth medium. (C) *bmoX*::*lacZ* reporter strain grown on 1-butanol (3 mM) under anoxic conditions with KNO_3 (30 mM) added to the growth medium. Inocula were grown under the conditions described for each panel. The data points are representative of the means of three replicates, and the error bars are proportional to the standard deviations of the means.

the growth medium revealed that 0.5 μM AgCl was as effective as $CuSO_4$ at repressing β-galactosidase induction (data not shown). Repression by Ag^{1+} was observed only under ambient O_2 and not under low-O_2 conditions; Zn^{2+}, Cd^{2+}, Hg^{2+}, and Pt^{2+} had no effect on induction. All subsequent experiments were limited to $CuSO_4$, since it is an essential component of the standard growth medium.

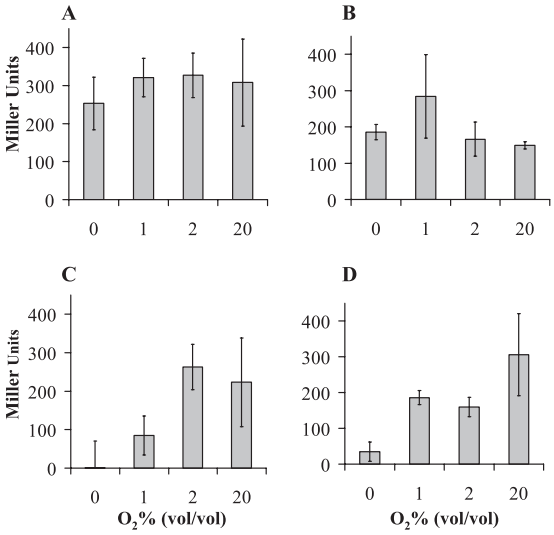

FIG. 2. Influences of buffer and growth medium mineral salts solution on the effects of percent O_2 and KNO_3 on 1-butanol-dependent induction of β-galactosidase activity in the *bmoX*::*lacZ* reporter strain of *P. butanovora*. Lactate-grown cells were washed and resuspended in NH_4Cl-NaK phosphate buffer (A), NH_4Cl-NaK phosphate buffer plus 30 mM KNO_3 (B), NH_4Cl-NaK phosphate-buffered complete mineral salts solution (C), and NH_4Cl-NaK phosphate-buffered complete mineral salts solution plus 30 mM KNO_3 (D). The values represent the means of three replicates, and the error bars represent the standard deviations of the means.

Effects of ascorbate and $CuSO_4$ on 1-butanol-dependent induction of β-galactosidase in the *bmoX*::*lacZ* reporter strain under oxic conditions. We examined the effect of the Cu^{2+} reducing agent ascorbate on 1-butanol-dependent β-galactosidase induction in the lactate-grown *bmoX*::*lacZ* reporter strain under oxic conditions. In NH_4Cl-KNa phosphate buffer, 1-butanol induced β-galactosidase to similar levels (~300 Miller units) in the presence or absence of 100 μM Na ascorbate. In contrast, when $CuSO_4$ (0.5 μM) was mixed with 100 μM Na ascorbate and added to the induction assay, β-galactosidase activity was induced by 1-butanol to ~18% of the level (55 Miller units) that was observed in the absence of ascorbate. These data show that the Cu^{2+} reducing agent Na ascorbate enhanced the sensitivity of β-galactosidase repression to $CuSO_4$ under oxic conditions.

TABLE 1. Effects of percent O_2 and transition metals on 1-butanol-dependent induction of β-galactosidase activity in lactate-grown cells of the *bmoX*::*lacZ* reporter strain of *P. butanovora*

Test element (0.5 μM)	β-Galactosidase activity (Miller units)[a]	
	20% O_2	1% O_2
Buffer	310 (41)[a]	283 (29)
Mn	303 (23)	288 (21)
Fe	322 (15)	304 (33)
Co	306 (31)	346 (28)
Ni	321 (31)	318 (12)
Cu	294 (45)	80 (25)
Zn	256 (25)	311 (43)

[a] The values represent the means of three replicates, and the standard deviations are provided in parentheses.

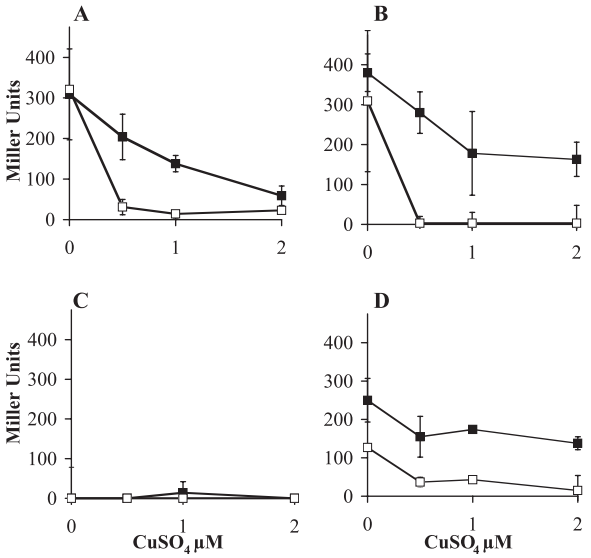

FIG. 3. Effects of O_2 and $CuSO_4$ on 1-butanol-dependent induction of β-galactosidase activity in lactate-grown (A and C) and butyrate-grown (B and D) *bmoX*::*lacZ* and R8-*bmoX*::*lacZ* reporter strains of *P. butanovora*. Experiments were conducted in NH_4Cl-KNa phosphate buffer in the presence of 20% O_2 (closed symbols) or 1% O_2 (open symbols). (A and B) The lactate- and butyrate-grown *bmoX*::*lacZ* reporter strains, respectively. (C and D) The lactate- and butyrate-grown BmoR-deficient R8-*bmoX*::*lacZ* reporter strains, respectively. The data points are the means of three replicates, and the error bars represent the standard deviations of the means.

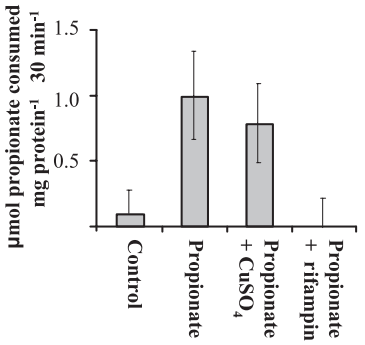

FIG. 4. Effects of $CuSO_4$ and rifampin on the induction of propionate consumption under 1% O_2. Lactate-grown cells of wild-type *P. butanovora* were washed and resuspended in NH_4Cl-KNa phosphate-buffered complete mineral salts solution under 1% O_2. Propionate (2 mM), rifampin (40 μg/ml), or $CuSO_4$ (0.5 μM) was added as indicated. The values represent the means of three replicates, and the error bars represent the standard deviations of the means.

Expression of a substrate-dependent, BmoR-independent BMO regulatory system influences the sensitivity of 1-butanol-dependent induction of β-galactosidase to $CuSO_4$ repression under oxic conditions. As mentioned above, we recently identified a putative σ^{54} factor (BmoR) that was hypothesized to be involved in expression of the *bmo* operon (15). Although β-galactosidase activity could not be induced by 1-butanol in lactate-grown cells of a BmoR-deficient variant of the *bmoX*::*lacZ* reporter strain (the R8-*bmoX*::*lacZ* strain), 1-butanol partially induced β-galactosidase activity in the R8-*bmoX*::*lacZ* strain after growth on propionate, butyrate, or valerate (15). These data suggested the existence of a second, BmoR-independent BMO regulatory system. In this work, we describe further studies with the two *lacZ* reporter strains (the *bmoX*::*lacZ* and R8-*bmoX*::*lacZ* strains) that illustrate that the 1-butanol-dependent induction of BMO in butyrate-grown cells is less sensitive to repression by $CuSO_4$ than in lactate-grown cells, regardless of whether BmoR is functional or inactive. As shown in Fig. 3A, $CuSO_4$ (2 μM) almost completely repressed 1-butanol-dependent induction of β-galactosidase under oxic conditions in lactate-grown cells of a BmoR-positive *bmoX*::*lacZ* strain (Fig. 3A). In butyrate-grown cells, however, about 50% of the 1-butanol-induced β-galactosidase activity (~180 to 200 Miller units) was insensitive to repression by up to 2 μM $CuSO_4$ (Fig. 3B). An identical experiment was conducted with the BmoR-deficient R8-*bmoX*::*lacZ lacZ* reporter strain (Fig. 3C and D). In the case of lactate-grown cells, β-galactosidase activity was not induced by 1-butanol regardless of the presence or absence of $CuSO_4$ and confirmed the essentiality of BmoR for the induction of lactate-repressed cells (Fig. 3C). In the case of butyrate-grown cells, however, 1-butanol induced β-galactosidase activity to about one-half of the level (~250 Miller units) induced in the BmoR-positive *bmoX*::*lacZ* reporter strain under oxic conditions (Fig. 3B versus D), and about 60 to 75% of this activity (150 to 180 Miller units) was insensitive to repression by 2 μM $CuSO_4$ (Fig. 3D). In contrast, under 1% O_2, 1-butanol-dependent induction of β-galactosidase activity in butyrate-grown cells of both the BmoR-positive (Fig. 3B) and the BmoR-deficient (Fig. 3D) reporter strains was completely or almost completely repressed by $CuSO_4$. These data suggest that the BmoR-independent regulation of the BMO promoter is less sensitive to $CuSO_4$ under oxic conditions but remains quite sensitive to 0.5 μM $CuSO_4$ under low-oxygen or anoxic conditions. The lack of $CuSO_4$-resistant induction in the lactate-grown *bmoX*::*lacZ* reporter strain (Fig. 3A) implies that the BmoR-independent system is not expressed during growth on lactate. At this time, it is unknown if both BMO regulatory systems are expressed in butyrate-grown cells.

Effects of $CuSO_4$ on growth, O_2 consumption, and the induction of propionate consumption in lactate-grown *P. butanovora*. Several experiments were conducted to determine if $CuSO_4$ repression of 1-butanol-dependent induction of BMO might be caused by general copper toxicity on *P. butanovora*. Generally, there was no evidence of copper toxicity at concentrations that are effective in the repression of BMO. First, although *P. butanovora* did not grow in medium without supplemental $CuSO_4$, it grew on butyrate as a sole C source in the presence of 2 μM supplemental $CuSO_4$ at a rate comparable to that achieved in normal growth medium (~0.2 μM $CuSO_4$). Second, endogenous and 1-butanol-dependent O_2 consumption rates were both unaffected by exposure of cells to $CuSO_4$ at ≤2 μM. Third, $CuSO_4$ (≤2 μM) had no significant inhibitory effect on preexisting BMO activity as determined on butane-grown cells treated with chloramphenicol to stop further protein synthesis. Fourth, the induction of propionate consumption under 1% O_2 was unaffected by the presence of 0.5 μM $CuSO_4$ (Fig. 4), suggesting that the integrated processes of transcription and translation associated with the development

of a catabolic pathway in *P. butanovora* were insensitive to $CuSO_4$ at concentrations that affected BMO induction.

DISCUSSION

The results of this study clearly demonstrate that *P. butanovora* has a previously uncharacterized ability to repress transcription from the BMO promoter when O_2 is limiting. BMO repression occurs even in the presence of an inducer (e.g., 1-butanol), and copper ions play a critical role. Copper was an effective repressor at low concentrations ($<\sim$0.2 to 2 μM), similar to those used by researchers of the well-studied "copper switch" phenomenon of copper repression of sMMO in methanotrophs (4, 11, 19, 22), an enzyme genetically, biochemically, and structurally related to BMO (3, 13, 26). The fact that the repressive effect of copper occurs at lower concentrations of copper ions under anoxic than under oxic conditions and that the presence of the Cu^{2+} reducing agent ascorbate enables lower concentrations of copper ions to be effective under oxic conditions indicates that regulation by copper ions is linked to a redox-sensitive system.

Recent research from our laboratory identified a σ^{54}-type transcriptional regulator, BmoR, that is encoded by a gene upstream of the *bmo* operon and that is required for complete alcohol-dependent induction of BMO by lactate- and acetate-grown *P. butanovora*, but not for alcohol-dependent induction by propionate-, butyrate-, or valerate-grown cells (15). Data presented in the study unequivocally show that the copper repression of 1-butanol-dependent induction occurs under both oxic and anoxic incubation conditions, regardless of whether cells are grown on lactate or butyrate and whether or not BmoR is actively involved in the regulation of BMO expression. Clearly, our research is in its infancy, and more work is needed to identify the molecular mechanisms associated with BmoR-dependent and -independent induction of BMO. Interestingly, a substantial percentage of the induced activity is copper insensitive in butyrate-grown cells and an even larger percentage in the absence of a functional BmoR. Given the relatively large errors associated with analyzing β-galactosidase activity in replicate samples, further detailed work is needed to more accurately determine the size of the fraction of activity that is copper insensitive, particularly in the BmoR-deficient mutant.

There is some precedent in the literature for σ^{54}-type transcriptional regulators to be able to function through redox mechanisms. For example, up-regulation of the nitrogenase (*nif*) operon in many diazotrophic bacteria under anoxic conditions is controlled by the activity of a σ^{54} promoter under the control of the redox-influenced NifA/NifL proteins (5, 14, 16). Recently, Ukaegbu and colleagues proposed a model of copper repression of the sMMO operon in which the relative amounts of the oxidized and reduced forms of a regulatory protein, MmoS, are controlled by the level of Cu^{2+} ions. In the absence of Cu^{2+} ions, the reduced form of MmoS dominates and expresses a kinase activity that phosphorylates MmoR, which then up-regulates the transcription of sMMO (28). Obviously, much more work is needed to rigorously determine if the effect of copper ions on BMO expression follows a model similar to that proposed for sMMO. A particularly interesting aspect of the putative relationship between copper and redox in regulation will be the way in which ascorbate under oxic conditions, or anoxic conditions without ascorbate, can lower the effective concentration of copper ions required for BMO repression. For example, this could be achieved by anoxic conditions facilitating transformation of Cu^{2+} to Cu^{1+}, with the latter being the active ionic species. In this context, two points are worthy of note (1). We showed that Ag^{1+} is a potent repressor of BMO induction under oxic conditions, and in contrast to copper, Ag^{1+} is the normal oxidation state of silver found under oxic and anoxic conditions (2). It has been shown that methanobactin, the copper-binding molecule secreted by methanotrophs, binds Cu^{2+}, which is subsequently reduced to Cu^{1+} (2, 12). It remains unclear if Cu^{1+} plays any role in the regulation of sMMO.

In methanotrophs, copper ions are also involved in up-regulation of genes encoding the copper-containing protein particulate MMO. By analogy, copper might be involved in up-regulating genes encoding copper-containing proteins needed for growth of *P. butanovora* on alcohols under anoxic nitrate-respiring conditions. For example, there are at least two 1-butanol dehydrogenases in *P. butanovora*, one of which is a quinohemoprotein (29), and electron transport from the latter type of alcohol dehydrogenase often occurs via a copper-containing protein, azurin (10, 17). A blue redox-sensitive azurin-like protein has been detected in extracts of *P. butanovora* (L. A. Sayavedra-Soto and D. J. Arp, unpublished data). Furthermore, the copper ion is an essential cofactor in two of the enzymes involved in nitrate respiration (NirK and NosZ) under anoxic conditions (31). Although we do not know if *P. butanovora* contains a NirK- or NirS-like nitrite reductase, its growth under denitrifying conditions is inhibited by the copper chelator allylthiourea and by acetylene, a potent inhibitor of Cu containing nitrous oxide reductase (NosZ). Finally, we know that cells of *P. butanovora* grown under denitrifying conditions do not induce BMO in response to a short incubation with alcohols under oxic conditions, indicating that the alcohol-dependent inducing system of BMO is inactivated after anoxic metabolism becomes established (Doughty et al. unpublished). It is known that facultative aerobes, such as *Escherichia coli* and *Pseudomonas aeruginosa*, possess molecular regulatory systems that enable them to switch from aerobic to anoxic growth by nitrate respiration under O_2-limited conditions (9, 21, 25). More work is needed to determine how the copper ion repression of BMO induction is linked to the molecular mechanisms of regulation that permit *P. butanovora* to transition from oxic to anoxic growth conditions and what is involved in its inactivation.

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