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Cytochrome *bd* Oxidase, Oxidative Stress, and Dioxygen Tolerance of the Strictly Anaerobic Bacterium *Moorella thermoacetica*

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The gram-positive, thermophilic, acetogenic bacterium *Moorella thermoacetica* can reduce CO_2 to acetate via the Wood-Ljungdahl (acetyl coenzyme A synthesis) pathway. This report demonstrates that, despite its classification as a strict anaerobe, *M. thermoacetica* contains a membrane-bound cytochrome *bd* oxidase that can catalyze reduction of low levels of dioxygen. Whole-cell suspensions of *M. thermoacetica* had significant endogenous O_2 uptake activity, and this activity was increased in the presence of methanol or CO, which are substrates in the Wood-Ljungdahl pathway. Cyanide and azide strongly (~70%) inhibited both the endogenous and CO/methanol-dependent O_2 uptake. UV-visible light absorption and electron paramagnetic resonance spectra of *n*-dodecyl-β-maltoside extracts of *M. thermoacetica* membranes showed the presence of a cytochrome *bd* oxidase complex containing cytochrome b_{561}, cytochrome b_{595}, and cytochrome *d* (chlorin). Subunits I and II of the *bd* oxidase were identified by N-terminal amino acid sequencing. The *M. thermoacetica* cytochrome *bd* oxidase exhibited cyanide-sensitive quinol oxidase activity. The *M. thermoacetica* cytochrome *bd* (*cyd*) operon consists of four genes, encoding subunits I and II along with two ABC-type transporter proteins, homologs of which in other bacteria are required for assembly of the *bd* complex. The level of this *cyd* operon transcript was significantly increased when *M. thermoacetica* was grown in the absence of added reducing agent (cysteine + H_2S). Expression of a 35-kDa cytosolic protein, identified as a cysteine synthase (CysK), was also induced by the nonreducing growth conditions. The combined evidence indicates that cytochrome *bd* oxidase and cysteine synthase protect against oxidative stress and contribute to the limited dioxygen tolerance of *M. thermoacetica*.

Dioxygen is the preferred electron sink in the respiratory chain of most aerobic bacteria (58). Cytochrome oxidases are the membrane-bound, terminal components of the dioxygen-dependent respiratory chain, which reduces dioxygen to water with formation of pH and potential gradients (6, 45). Two main types of respiratory cytochrome oxidases are known in bacteria: the heme/copper oxidases (cytochrome aa_3, cytochrome caa_3 oxidase, and cytochrome *bo* oxidase), and the heme-only cytochrome *bd* quinol oxidase, which is associated with microaerobic dioxygen respiration (24, 56, 59, 62). Cytochrome *bd* oxidases purified from aerobic bacteria have been characterized as 1:1 heterodimers of two integral membrane proteins referred to as subunits I and II (35, 55). The heterodimers contain three heme components: a low-spin heme, b_{558}, and two high-spin hemes, b_{595} and *d*. In the genomes of *Escherichia coli* (31) and *Azotobacter vinelandii* (41), subunits I and II are encoded by *cydA* and *cydB*, respectively, of the cytochrome *bd* (*cyd*) operon. Two ABC-type transporter proteins, CydD and CydC, are typically required for assembly of the cytochrome *bd* complex in both gram-positive and gram-negative aerobic bacteria (14, 60). Aerobic respiration not only conserves energy but also generates reactive oxygen species, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical, which are known to damage membrane lipids, proteins, DNA, and iron-sulfur clusters (3, 4, 28, 34, 43).

Strictly anaerobic bacteria would seem to have no need of respiratory cytochrome oxidases. Nevertheless, functional cytochrome *bd*-type oxidases have recently been discovered in bacteria classified as strict anaerobes: e.g., *Bacteroides fragilis* (2) and *Desulfovibrio gigas* (50). The gram-positive, thermophilic bacterium *Moorella thermoacetica* (formerly *Clostridium thermoaceticum*) is an obligately anaerobic acetogen that can grow autotrophically on CO_2/H_2 or CO via the Wood-Ljungdahl (acetyl coenzyme A [CoA]) pathway (21). This pathway conserves energy via chemiosmosis from a membrane-bound electron transport chain consisting of two *b*-type cytochromes (b_{559} and b_{554}), and a menaquinone, MK7 (16, 19). Although classified as strict anaerobes, several acetogenic bacteria, including *M. thermoacetica*, have been reported to tolerate traces of dioxygen (7, 8, 39, 48). Karnholz et al. showed that *M. thermoacetica* can survive exposure to and consume low levels (0.5 to 2%) of headspace dioxygen in liquid culture and also reported NADH oxidase activity in cell extracts (39). Subsequently, we discovered a five-gene cluster in *M. thermoacetica* encoding oxidative and nitrosative stress protection proteins (20, 66). During the course of these studies, we found that the consumption of dioxygen by *M. thermoacetica* is inhibited by cyanide, a strong inhibitor of cytochrome oxidases. Accordingly, we have identified a cytochrome *bd*-type oxidase exhibiting menaquinol oxidase activity in membranes of *M. thermoacetica*. Subsequent genome sequence data and Northern blotting confirmed the presence of a cytochrome *bd* oxidase operon in *M. thermoacetica*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. thermoacetica* strain ATCC 39073 was grown on 200 mM methanol as the carbon source at 58°C under 100% CO_2 gas in semidefined Drake's minimal medium (65) in 125-ml bottles, 4-liter flasks,

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or a 100-liter fermentor in either the presence or absence of reducing agent (Na_2S, 0.5 g/liter; cysteine HCl, 0.5 g/liter) as previously described (18, 20). *E. coli* strain DH5α was grown on glucose (20 mM) at 37°C in 4-liter flasks in MZ9 salt medium (17, 20). Cultures were harvested at mid- to late log phase (optical density at 600 nm [OD_{600}] of ~1.0) by centrifugation at 6,000 × *g* and stored at −80°C until used. Cells used in O_2 uptake experiments were harvested under anoxic conditions as described below.

Membrane preparation. Membranes of *M. thermoacetica* were prepared from whole cells by a combination of lysozyme and French press methods, and *E. coli* membranes were prepared after breaking cells with a French press as described previously (17).

Membrane extracts and partial purification of cytochrome *bd* oxidase. Crude membranes were washed in 50 mM Tris-HCl, pH 8.0, and then suspended (1.0 mg/ml) in 10 mM Tris (Tris)-HCl, pH 8.0, 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (PMSF) and incubated at 4°C overnight to remove F_1-ATPase, which otherwise coextracted with cytochrome *bd* oxidase. Solubilization and partial purification of cytochrome *bd* oxidase from washed membranes were carried out according to Junemann and Wrigglesworth (36) with some modifications. F_1-ATPase-depleted membranes were suspended in buffer A (potassium phosphate, pH 7.5, 1 mM EDTA, pH 8.0, 1 mM PMSF) to a protein concentration of 5 mg/ml. Sodium cholate was added to a final concentration of 1.5% (wt/vol), and the suspension was stirred at 4°C overnight. After centrifugation at 100,000 × *g* (1 h), the supernatant, containing loosely bound membrane protein and a small amount of cytochrome *bd* oxidase, was discarded. The pellet containing the majority of the cytochrome *bd* oxidase was suspended in buffer A at a protein concentration of 5 mg/ml, and *n*-dodecyl-β-maltoside (DM) (Anatrace, Maumee, Ohio) was added to a final concentration of 1% (wt/vol). The suspension was stirred at 4°C for 2 h and then centrifuged as described above. The greenish-yellow supernatant containing cytochrome *bd* oxidase was stored at −80°C until further use. This fraction, referred to as DM extracts, was concentrated, if necessary, by Amicon ultrafiltration and used in all spectroscopic studies. To further purify the enzyme, DM extracts (15 to 20 ml and 4 to 5 mg of protein per ml) were dialyzed against 1 liter of buffer A without EDTA but containing 0.02% DM and then loaded onto a 5-ml hydroxyapatite column (CHT column; Bio-Rad, Hercules, Calif.). The column was washed with the same buffer. Bound cytochrome *bd* oxidase was eluted from the column first with 0.4 M and then with 0.8 M potassium phosphate containing 0.02% DM. Most of the cytochrome *bd* oxidase was eluted by 0.8 M potassium phosphate, as confirmed by spectroscopic analysis.

DNA and RNA sources, PCR, and Northern hybridization. *M. thermoacetica* genomic DNA was isolated with the Puregene DNA purification system (Gentra, Minneapolis, Minn.). Total RNA was isolated with the RNeasy mini kit (QIAGEN, Inc., Valencia, Calif.). Prior to gel electrophoresis and hybridization experiments, total RNA was treated with RNase-free DNase I (Roche Applied Sciences, Indianapolis, Ind.). For dot blot hybridization experiments RNA was denatured with formaldehyde (15% [vol/vol]) in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 60°C for 1 h prior to application onto nylon membranes (ICN, Costa Mesa, Calif.). Hybridization (Northern or dot blot) experiments were carried out with the Genius system (Roche Applied Sciences) as described previously (15, 20). Digoxigenin (DIG)-labeled PCR products used as probes in hybridization experiments were amplified with *M. thermoacetica* genomic DNA by using the FailSafe PCR system (EPICENTRE, Madison, Wis.). The following PCR products were used as probes for hybridization experiments: a 336-bp fragment belonging to *cydA* amplified with 5′-GATTTCTTCG CCCTACTA-3′ (forward primer; Fp) and 5′-TGTTTTGACTGGTTTTCCT-3′ (reverse primer; Rp); a 320-bp fragment belonging to the β-subunit-gene of the CODH operon (accession no. M62727) amplified with 5′-ATTATAGCGTCAA GGACGA-3′ (Fp) and 5′-ACAGGGTCGTTATCCATA-3′ (Rp); a 355-bp fragment belonging to *cysK* (cysteine synthase K) encoding a 35-kDa protein (see Results); and a 390-bp fragment belonging to the β-subunit gene (*atpD*) of the F_1-ATP synthase operon amplified with primers as described previously (15).

Native PAGE and heme staining. Native polyacrylamide gel electrophoresis (PAGE) (without sodium dodecyl sulfate [SDS]) of DM extracts of membranes was carried out according to Laemmli (49) in the presence of 0.1% DM in the resolving gel. The gels were stained for heme with 3,3′,5,5′-tetramethylbenzidine (TMBZ) according to the method described by Thomas et al. (70). After staining for heme, the same gel was destained and then restained for the detection of protein with Coomassie brilliant blue. The Coomassie-stained protein band corresponding to the heme complex was excised from the native gel and subjected to SDS-PAGE (64).

Antibodies and Western blotting. Polyclonal antibodies against a peptide designed from the highly conserved carboxyl-terminal amino acid sequence (Q302-V318) of *E. coli* CydD (14) were a gift from Robert K. Poole of the University of Sheffield, Sheffield, United Kingdom. Western blotting experiments were carried out according to Bio-Rad (17).

N-terminal peptide sequences. Partially purified *M. thermoacetica* cytochrome *bd* oxidase was subjected to SDS-PAGE and then trans-blotted onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Resolved proteins were briefly stained with Coomassie brilliant blue and then excised from the blots. The N-terminal amino acid sequences were determined either in the Integrated Biotechnology Laboratories at the University of Georgia or in the Microchemical and Proteomics Facility at the Emory University School of Medicine.

Spectroscopic measurements. UV-visible absorption spectra were recorded with a dual-wavelength Shimadzu, Inc., model 2051PC spectrophotometer. Cytochromes were reduced chemically with a few grains of sodium dithionite or enzymatically with reduced duroquinone (DQH_2) or reduced ubibiquinone-1 (QH_2). Quinols were prepared from duroquinone or ubiquinone-1 (from Sigma Chemical Co.) by reduction with dithionite as described previously (63). The reductions of cytochromes by quinols were carried out anoxically in a 1-cm-path-length quartz cuvette, under 100% argon. Electron paramagnetic resonance (EPR) spectra were recorded with a Bruker ESP-300E spectrometer equipped with an ER-4116 dual-mode cavity and an Oxford Instruments ESR-flow cryostat (20) under conditions given in the figure legend.

Assays and activity measurements. All operations using whole cells were carried out under anoxic conditions in a Coy chamber (Vacuum Atmospheres Company, Hawthorne, Calif.) containing an atmosphere of 5% H_2, 10% CO_2, and 85% N_2 by volume; those using membranes or DM extracts were carried out under oxic (atmospheric dioxygen) conditions. For O_2 uptake measurements, washed membranes or cells were suspended to 50 to 100 μg of protein/ml in 2 ml of 50 mM morpholinepropanesulfonic acid (MOPS; pH 7.0) for membranes and 20 mM potassium phosphate pH 7.0 (sparged with N_2 gas) for cell suspensions. Dioxygen uptake experiments were carried out at room temperature (~25°C) in the Coy anoxic chamber, using a Clarke-type oxygen electrode calibrated with air-saturated and N_2-saturated buffer as described previously (39). The initial dioxygen concentrations used for the measurement of O_2 uptake by whole-cell suspensions were adjusted to 15 to 20 μM by injecting appropriate volumes of air into the headspace of the culture bottles. Anoxic stock solutions of all substrates and inhibitors were prepared under 100% N_2 gas prior to injection into assay mixtures. Quinol oxidase activity of cytochrome *bd* oxidase was determined in 100 mM potassium phosphate buffer (pH 7.0) by measuring O_2 uptake according to Junemann and Wrigglesworth (36). The reaction mixture (2.0 ml) contained 100 μM quinols as substrate (DQH_2 or QH_2 prepared as described above). The reaction was initiated by adding 50 to 100 μg of protein from DM extracts or partially purified cytochrome *bd* oxidase that had been preincubated on ice for 5 min with 1 mM phospholipids, L-α-phosphatidylcholine type XV1-E (Sigma). The consumption of dioxygen was recorded at room temperature with the oxygen electrode as described above. For inhibition studies, cyanide was added to the reaction mixture along with the substrate (quinol) prior to the addition of the protein.

***M. thermoacetica* genome sequence.** A draft annotated nucleotide sequence of the *M. thermoacetica* (ATCC 39073) genome has been completed at the Joint Genome Institute, Department of Energy, and can be viewed at http://genome.jgi-psf.org/draft_microbes/mooth/mooth.draft.html.

RESULTS

The *M. thermoacetica* cytochrome *bd* oxidase operon. Although we had isolated the *M. thermoacetica* cytochrome *bd* oxidase prior to any knowledge of the genome sequence, the results are more logically presented by first establishing the existence and transcription of a cytochrome *bd* oxidase (*cyd*) operon. Figure 1 diagrams the composition of the 6,326-bp four-gene *M. thermoacetica cyd* operon, annotated as *cydABCD* (genes 1660 to 1663 on contig 300 of the *M. thermoacetica* genome sequence). The deduced amino acid sequences of the two genes annotated as *cydA* and *cydB* are homologous to those of CydA (subunit I) and CydB (subunit II), respectively, of the cytochrome *bd*-type quinol oxidases from *E. coli* (43 and 40% identities, respectively) and *B. subtilis* (53 and 46% identities, respectively). A hydropathy plot of the predicted amino acid sequence of *M. thermoacetica* CydA (not shown) indicates nine membrane-spanning regions, the locations of which are

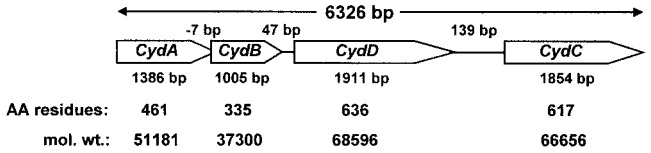

FIG. 1. Composition of the *M. thermoacetica* cytochrome *bd* oxidase operon (*cydABDC*) and the relationship between the genes and their products. The direction of transcription is from left to right. AA, amino acid; mol. wt., molecular weight.

very similar to those of CydA from *E. coli* (77), with the exception of some minor differences at the carboxyl-terminal end, due mostly to the absence of 56 residues from the *M. thermoacetica* sequence (Gly-322 to Met-377 of *E. coli* CydA) (cf. Fig. 2); these residues are also missing in CydAs of other gram-positive bacteria (e.g., *Bacillus subtilis*; NCBI accession no. A69611). Three of the four axial ligands to the irons of the three hemes have been identified as His-19, His-186, and Met-393 of CydA (subunit I) in the *E. coli* cytochrome *bd* oxidase (25, 40, 68, 69). The corresponding residues are conserved as His-18, His-183, and Met-333 in *M. thermoacetica* CydA. A highly conserved sequence, GWXXXEXGRQPW, near the quinol-binding site of *E. coli* CydA (76) is also conserved in *M. thermoacetica* CydA, except that Q is replaced by Y. Hydropathy plots of the *E. coli* and *M. thermoacetica* CydB amino acid sequences (data not shown) are also very similar to each other.

The amino acid sequences of the proteins encoded by *cydD* and *cydC* of the *M. thermoacetica cyd* operon are homologous to ABC-type membrane transporters, CydD and CydC, respectively, from *E. coli* (35.8 and 36.2% identities, respectively) and *B. subtilis* (32.4 and 32.4% identities, respectively). Both CydD and CydC are required for the biosynthesis and assembly of the cytochrome *bd* complex in *E. coli* (60). Four-gene *cydABDC* operons similar to that of *M. thermoacetica* are found in *Mycobacterium tuberculosis* (13), *Lactobacillus lactis* (NCBI accession no. NC_002662), *B. subtilis* (75), and *Listeria innocua* (NCBI accession no. NC_003212), whereas *cydD* and *cydC* are found in separate loci from *cydAB* in the *E. coli* and *A. vinelandii* genomes (31, 41).

The structural genes encoding subunit I (CydA) and subunit II (CydB) of *M. thermoacetica* cytochrome *bd* oxidase overlap by 7 bp (cf. Fig. 1), a feature often referred to as translational coupling of functionally related genes (47). A putative promoter sequence, 5′-CTTGAAA-N_{23}-TATACT-3′, resembling the *E. coli* consensus σ^{70} promoter (5′-cCTTGACa-$N_{15\text{-}21}$-TA TAaT-3′; lowercase type indicates nucleotides that are not strictly conserved) (33) is present 46 bp upstream of the *cydA* start codon. No secondary promoter was apparent in the two intergenic regions, between *cydB* and *cydD* or between *cydD*

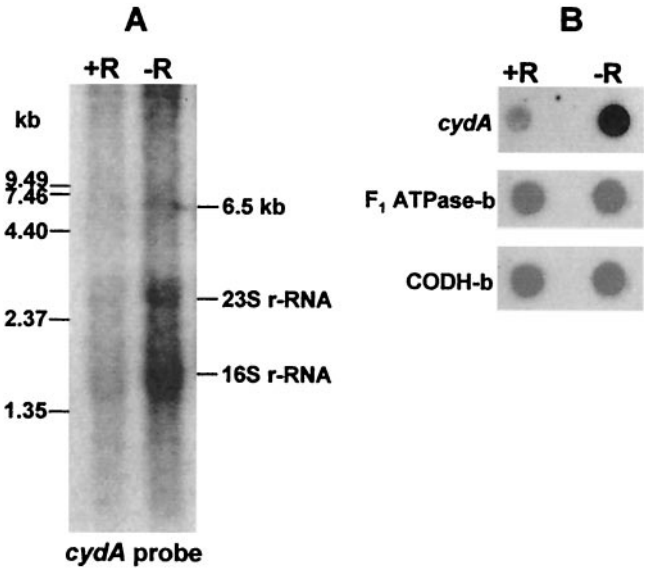

FIG. 3. (A) Northern blot of total RNA (5 μg in each lane) isolated from *M. thermoacetica* grown on methanol in the presence (+R) or absence (−R) of reducing agent after hybridization with a DIG-labeled DNA fragments amplified from *cydA* (A) of the *M. thermoacetica cyd* operon. The positions of RNA size standards are indicated on the left. The probe also hybridized nonspecifically to 23S (2.928 kb) and 16S (1.553 kb) rRNA. (B) RNA dot blots of *M. thermoacetica* total RNA (each spot loaded with 5 μg of DNase-treated RNA) after hybridization with the DIG-labeled DNA fragment amplified from *cydA*, the F_1-ATPase β-subunit gene (*atpD*), and the CO dehydrogenase β-subunit gene.

and *cydC*, suggesting that the *M. thermoacetica cyd* operon is transcribed as a single polycistronic message. To verify this presumption, total RNA from *M. thermoacetica* was subjected to Northern hybridization and probed with a 336-bp DIG-labeled DNA fragment amplified from *cydA*. Northern blots containing RNA from cells grown in nonreducing medium (i.e., omitting addition of cysteine and H_2S to the growth medium) showed a weak signal corresponding to an approximate size of 6.5 kb (lane −R, Fig. 3A). The 6.5-kb band is close to the predicted size of the *M. thermoacetica cyd* operon, 6,326 kb. A smaller-size smear starting at ~3.5 kb indicates degradation of the *cyd* transcripts. In addition to *cyd*-specific transcripts, the *cydA* probe also hybridized nonspecifically to 16S and 23S rRNA; this nonspecific hybridization may have been aided by the overlaps of the rRNA bands with the degraded *cydA* transcripts. These features, including degradative smearing and nonspecific hybridization to rRNA, were also observed on Northern blots of *B. subtilis cyd* transcripts (75). A similar hybridization signal was obtained in Northern blots of *M. ther-*

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MtCydA  313    ELQAAAAEQY GP..... ..... ..... ..... ..... .....GN YIPPVAPVFW
BsCydA  314    TLQAEYEKIY GK..... ..... ..... ..... ..... .....GD YIPPVKTTFW
MyCydA  324    DLQQEYQQRF GP..... ..... ..... ..... ..... .....ND YRPNLFVTYW
EcCydA  310    NLMAETYPRL QRGRMAWLLM QEISQGNREP HVLQAFRGLE GDLGYGMLLS RYAPDMNHVT AAQYQAAMRG AIPQVAPVFW
AvCydA  311    DLIADHEARI RNGMVRYGLL EELRAGNKSP EKIAAFNEVK DDLGYGLLLK KYTPNVVDAS EEQIKQAAKD TIPSVASMFW
VcCyDA  323    DLRDEHVERI RTGIYAYDLL ERLRAGEKTP ENMAAFDEVK HDLGYGLLLK RYTDKVTDAT EEQIQAAADD SIPTVWPLFW
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FIG. 2. Alignment of the deduced amino acid sequences at the COOH-terminal end of CydAs from *M. thermoacetica* (MtCydA), *B. subtilis* (BsCydA; accession no. A69611), *M. tuberculosis* (MyCydA; accession no. E70558), *E. coli* (EcCydA; accession no. S17958), *A. vinelandii* (AvCydA; accession no. A38170), and *V. cholerae* (VcCydA; accession no. E82149).

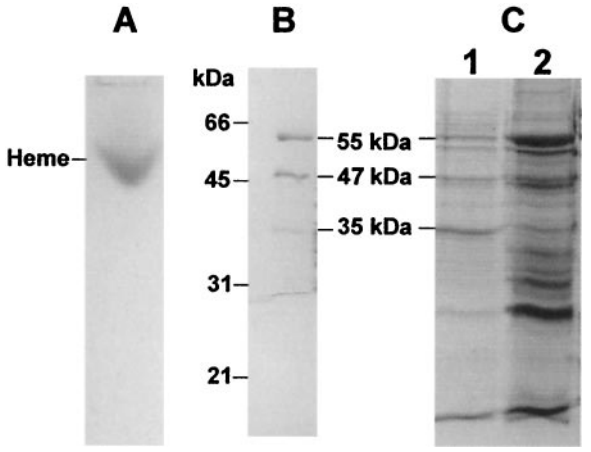

FIG. 4. PAGE and SDS-PAGE of *M. thermoacetica* cytochrome *bd* oxidase. (A) Native PAGE (8% acrylamide in resolving gel) of DM extracts (40 μg of protein) after heme staining with TMBZ. (B) SDS-PAGE of the heme-positive band excised from the native gel shown in panel A after staining with Coomassie brilliant blue. (C) SDS-PAGE of DM extracts (lane 2, 40 μg of protein) and partially purified cytochrome *bd* oxidase (lane 1, 20 μg of protein).

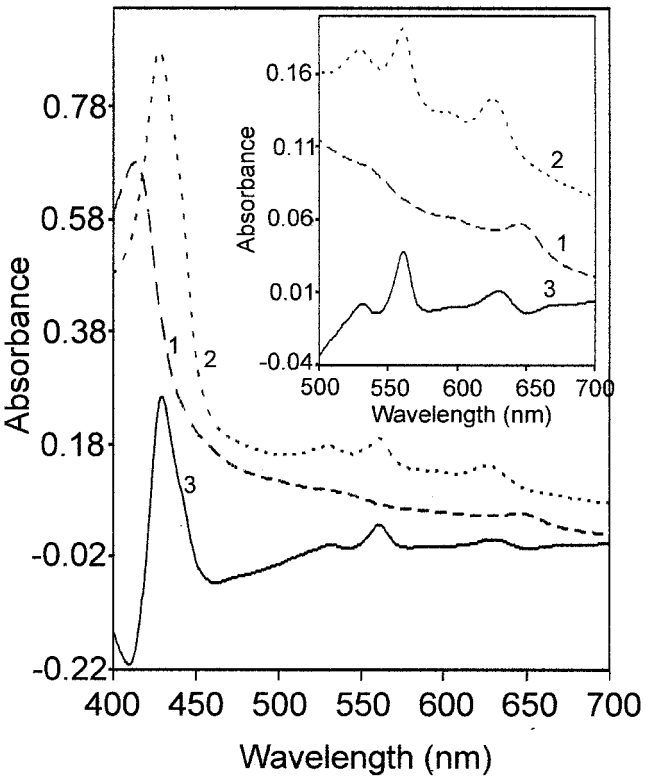

FIG. 5. UV-visible absorption spectra of *M. thermoacetica* cytochrome *bd* oxidase. The spectra were recorded on DM extracts (4.5 mg of protein/ml) of cholate-washed *M. thermoacetica* membranes in 0.1 M potassium phosphate (pH 7.5) and 1 mM EDTA. Trace 1 is air oxidized, trace 2 is dithionite reduced, and trace 3 is dithionite reduced minus air oxidized.

moacetica RNA probed with a PCR-amplified, DIG-labeled DNA fragment from *cydC* (data not shown), indicating that all four genes of the *M. thermoacetica cyd* operon are transcribed into a single polycistronic transcript, also as reported for *B. subtilis* (75). The Northern hybridization intensities corresponding to *cyd* transcripts were relatively stronger with RNA from cells grown in nonreducing (−R) than in reducing (+R) medium. Consistent with the Northern blot results, RNA dot blots (Fig. 3B) probed with the *cydA* fragment showed higher intensities from cells grown in nonreducing medium, whereas dot blot s probed for two "housekeeping" genes, encoding F_1-ATP synthase and CO dehydrogenase, each exhibited comparable +R and −R hybridization intensities.

Heme staining and identification of *M. thermoacetica cytochrome bd* oxidase subunits. DM extracts of *M. thermoacetica* membranes were subjected to native PAGE and heme staining. A single heme-positive band was found (Fig. 4A), which was resolved by SDS-PAGE into three major protein bands with approximate molecular masses of 35, 47, and 55 kDa (Fig. 4B). These sizes matched closely those of three of the four proteins from a partially purified cytochrome *bd* complex (Fig. 4C). The N-terminal amino acid sequences of the three proteins excised from the heme-stained gel were 35 kDa, MDL(I)XILWFILV; 47 kDa, XGQQKSGAEQ; and 55 kDa, A/MDAL/GLLA RWQ(G)F (where "X" denotes an unidentified residue and the letter in parentheses indicates a tentatively assigned residue). The experimentally determined N-terminal amino acid sequences of the 35- and the 55-kDa proteins matched the predicted N-terminal sequences of the *cydA* and *cydB* gene products, respectively, of the *M. thermoacetica cyd* operon (cf. Fig. 1). The N-terminal sequence of the 47-kDa protein matched that annotated as a branched-amino acid ABC transporter, the substrate-binding protein in the *M. thermoacetica* genome. This 47-kDa protein is not part of the *cyd* operon (cf. Fig. 1), and the role that this copurifying protein may play in *M. thermoacetica* cytochrome *bd* oxidase was not further investigated.

In order to verify CydD expression, DM extracts from *M. thermoacetica* cells grown in the absence of reducing agent were subjected to Western blotting with antibodies against a synthetic peptide, ^{302}QPLRDLGTFYHAKAQAV^{318}, designed from the carboxyl-terminal end of *E. coli* CydD (14). The amino acid sequence of this peptide shares 53% identical residues with the corresponding region, ^{288}LPLRLLGSRYH AGLAGV^{304}, of the *M. thermoacetica* CydD. The *E. coli* CydD peptide antibodies were found to react with a single protein of molar mass ~65 kDa in *M. thermoacetica* DM extracts (not shown). This mass is close to that predicted (68,596 Da, cf. Fig. 1) from the deduced amino acid sequence of *M. thermoacetica cydD*.

Spectroscopic analysis of *M. thermoacetica cytochrome bd* oxidase. Figure 5 shows UV-visible absorption spectra of DM extracts of *M. thermoacetica* membranes, both air oxidized (as-isolated) and dithionite reduced, as well as the dithionite-reduced − air-oxidized difference spectrum. The main features of these spectra included absorption peaks at 649 and 411 nm of oxidized samples; shifting of these peaks to 630 and 431 nm, respectively, upon reduction by dithionite; and the appearance of a prominent peak at 561 nm in dithionite-reduced samples. These spectra are very similar to corresponding spectra of purified cytochrome *bd* oxidases from several other bacteria

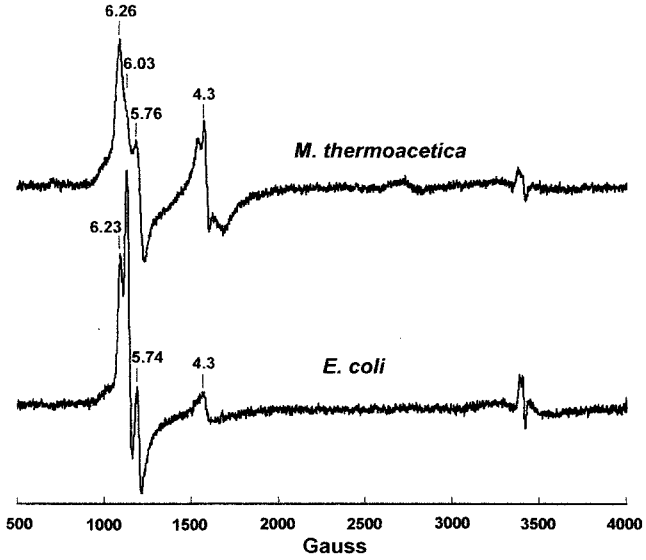

FIG. 6. EPR spectra of as-isolated cytochrome *bd* oxidases from *M. thermoacetica* and *E. coli*. DM extracts of *M. thermoacetica* or *E. coli* membranes (20 mg of protein/ml) in 50 mM Tris-HCl (pH 8.0) and 100 mM EDTA. EPR conditions were as follows: temperature, 4.0 K; microwave frequency, 9.602 GHz; microwave power, 2 mW; and modulation amplitude, 6.34 G.

(30, 36, 55). The absorption peaks at 561 and 630 nm from dithionite-reduced membranes correspond to heme b_{561} and heme *d* (chlorin), respectively, of cytochrome *bd* oxidase (36, 46). The reduced − oxidized absorption difference spectra of cytochrome *bd* oxidases from *E. coli* and *A. vinelandii* exhibit an absorption peak at 595 nm, corresponding to the third heme, b_{595}, of the enzyme complex (36, 46); the 595-nm absorption was rather weak in the difference spectrum of *M. thermoacetica* DM membrane extracts.

EPR spectra can also identify these cytochromes, and Fig. 6 shows that the EPR spectra of DM extracts from *M. thermoacetica* and *E. coli* are very similar to each other. The axial signal component at g = 6.03 (better resolved in the *E. coli* spectrum) corresponds to the high-spin ferric heme *d* (chlorin) and the rhombic signal components at g = 6.26 and 5.76 in the *M. thermoacetica* spectrum (or 6.23 and 5.74 in the *E. coli* spectrum) correspond to the high-spin ferric heme b_{595} (36, 53). The additional signals at g = 4.3 have been assigned to nonspecifically bound iron (54).

For simplicity, these DM extracts are, hereafter, referred to as cytochrome *bd* oxidase. Figure 7 compares the UV-visible absorption spectral changes of reduced *M. thermoacetica* versus *E. coli* cytochrome *bd* oxidases after treatment with CO. CO binds to reduced cytochrome *d* and induces characteristic spectral changes (30, 36, 38). The dithionite-reduced + CO − dithionite-reduced difference absorption spectrum of *M. thermoacetica* cytochrome *bd* oxidase revealed peaks at 417, 534, and 639 nm and troughs at 433, 443, and 629 nm, which closely resemble peaks at 417, 540, and 642 nm and troughs at 432, 445, and 624 nm in the spectrum of *E. coli* cytochrome *bd* oxidase. Similar spectra have been obtained for CO-treated cytochrome *bd* oxidases from other bacteria (30, 36, 38, 42).

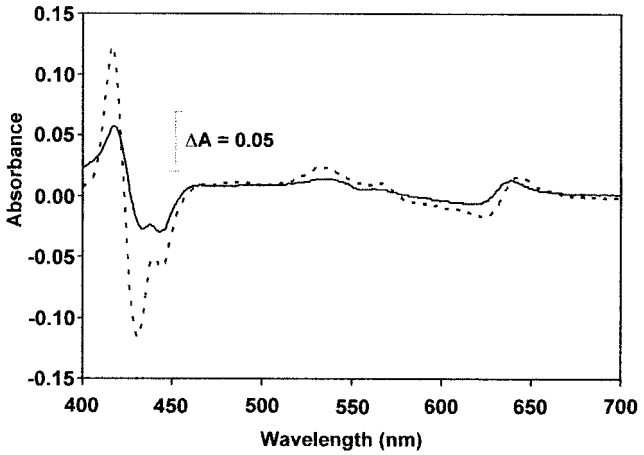

FIG. 7. Dithionite-reduced + CO − dithionite-reduced UV-visible absorption difference spectra of cytochrome *bd* oxidase in DM extracts (5.5 mg of protein/ml) of cholate-washed membranes from *M. thermoacetica* (solid trace) or *E. coli* (dashed trace).

Dioxygen uptake by whole cells and membranes. Several bacteria classified as strict anaerobes, including some acetogens, have been shown to tolerate and consume dioxygen (7, 8, 10, 11, 26, 27). *M. thermoacetica* cell suspensions showed significant consumption of O_2 in the absence of any exogenous electron donor (see footnote to Table 1), as has been observed in many other bacteria, including acetogens (7, 10, 26, 27). The rates of endogenous dioxygen consumption by *M. thermoacetica* cell suspensions grown in the presence (14.2 ± 3.6 nmol · min^{-1} · mg of $protein^{-1}$) or absence (29.8 ± 5.7 nmol · min^{-1} · mg $protein^{-1}$) of reducing agent were similar to those reported for other acetogenic bacteria (6). Exogenous methanol and CO, which are substrates in the Wood-Ljungdahl pathway, supported an increased O_2 uptake rate (Table 1), but these rates were much lower than those reported for other acetogens, e.g., *Sporomusa termitida*, *Acetonema longum*, or *Acetobacterium woodii* (7). These differences could be due to the wide variation of their origin and other physiological parame-

TABLE 1. Methanol- and CO-dependent O_2 uptake rates by *M. thermoacetica* cell suspensions[a]

Reducing agent[b]	Uptake rate (nmol · min^{-1} · mg of $protein^{-1}$)					
	CO (saturated)			Methanol (200 mM)		
	None	Azide	Cyanide	None	Azide	Cyanide
+Red	16.7	5.7	5.4	24.4	7.9	7.3
−Red	28.8	9.1	8.6	40.0	13.0	11.9

[a] Rates are listed after subtracting the following O_2 uptake rates in the absence of added CO or methanol: no inhibitor, 14.2 ± 3.6 (+Red) and 29.8 ± 5.7 (−Red) nmol · min^{-1} · mg of $protein^{-1}$ 2 mM azide, 5.4 ± 1.8 (+Red) and 11.0 ± 3.3 (−Red); nmol · min^{-1} · mg of $protein^{-1}$ and 1 mM KCN, 4.5 ± 1.4 (+Red) and 9.6 ± 3.0 (−Red) nmol · min^{-1} · mg of $protein^{-1}$. The listed values are averages of at least three separate experiments with standard deviations of 0 to 10%.

[b] Cultures were grown in Drake's medium (65) at 58°C under 100% CO_2 with methanol (200 mM) in the presence (+Red) or absence (−Red) of reducing agent. Cells were harvested anoxically at OD_{600} ≈1.0, washed, and resuspended to 50 to 100 μg of protein/ml anoxically in 20 mM potassium phosphate buffer (pH 7.0) containing either saturating CO (sparged for 15 min) or as described in Materials and Methods.

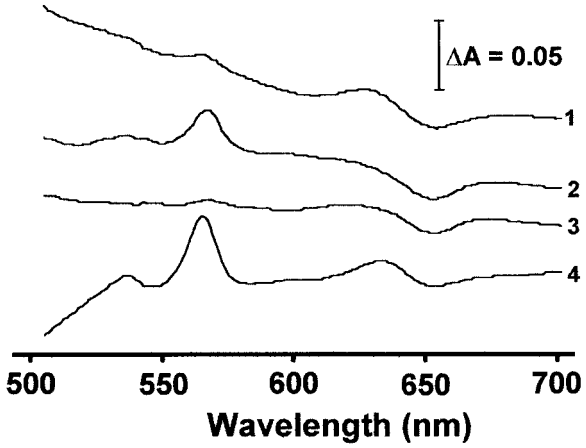

FIG. 8. UV-visible absorption difference spectra of *M. thermoacetica* cytochrome *bd* oxidase in DM extracts (6 mg of protein/ml). Traces: 1, DQH_2 reduced − air oxidized; 2, air oxidized + cyanide + DQH_2 − air oxidized + cyanide; 3, air oxidized + cyanide − air oxidized; 4, dithionite reduced − air oxidized.

ters (21). Both the endogenous and substrate-dependent O_2 uptake rates of *M. thermoacetica* cell suspensions were significantly higher for cells grown in the absence of reducing agent than those grown in its presence. Cyanide (1 mM) and azide (2 mM) inhibited both endogenous and substrate-dependent O_2 uptake up to 70% in whole-cell suspensions, indicating that most of the endogenous and exogenous substrate-dependent consumption of dioxygen follows an azide- and cyanide-sensitive pathway. Cyanide inhibition of O_2 consumption by whole cells has been reported in other bacteria, including acetogens (7, 37, 61).

NADH oxidase activity has been reported in cell extracts of several acetogens, including *M. thermoacetica* (39, 48), and Ivey (35) had reported NADH dehydrogenase activity and NADH-dependent cytochrome reduction in membranes of *M. thermoacetica*. The *M. thermoacetica* genome also encodes a putative NADH dehydrogenase complex. Accordingly, we found NADH-driven O_2 uptake activities in *M. thermoacetica* membranes: 7 nmol · min^{-1} · mg of $protein^{-1}$ from cells grown in the presence of reducing agent and 13 nmol · min^{-1} · mg of $protein^{-1}$ from cells grown in the absence of reducing agent. These membrane O_2 uptake activities were 50 to 55% inhibited by rotenone (60 μM), HQNO (50 μM), and cyanide (1 mM) and 37 to 40% inhibited by antimycin (5 μM), indicating that NADH-driven O_2 uptake by *M. thermoacetica* is coupled to a membrane electron transport chain.

Quinol oxidase activity. Quinols are the typical physiological electron donor for cytochrome *bd* oxidases. As shown in Fig. 8, traces 1 and 2, DQH_2 was able to reduce cytochrome *d* (detected as increased absorbance at 630 nm) in untreated *M. thermoacetica* cytochrome *bd* oxidase preparations but failed to do so in cyanide-pretreated samples (lower absorbance at 630 nm), consistent with the cyanide inhibition of O_2 uptake by whole cells and membranes presented above. Similar spectral changes were found with reduced ubiquinone-1 in place of DQH_2 (not shown). Comparison of the absorption peaks of b_{561} reduced by dithionite and quinols in Fig. 8 indicate incomplete reduction of cytochrome b_{561} by quinols. On the other hand, the levels of reduction of cytochrome *d* (absorption at 629 nm) by dithionite and quinol were comparable. These results suggest preferential reduction of *M. thermoacetica* cytochrome *d* over cytochrome b_{561} by quinols. Partial reduction of cytochrome b_{561} by quinols could be due to excess of the latter in the reaction mixture. Two *b*-type cytochromes, b_{554} and b_{559}, have previously been characterized from *M. thermoacetica* (16, 19). The *M. thermoacetica* genome also encodes two recognizable *b*-type cytochromes, b_{556} and b_{561}. Cytochrome b_{556} (encoded by gene 2271 of contig 306) belongs to a putative operon annotated as anaerobic-type dimethyl sulfoxide (DMSO) reductase, while cytochrome b_{561}, encoded by *cydA*, belongs to the *cyd* operon (cf. Fig. 1). The previously described cytochrome b_{554} thus apparently belongs to the DMSO reductase complex, while the cytochrome b_{559} should correspond to cytochrome b_{561} of cytochrome *bd* oxidase. We had shown previously that destruction of menaquinone (by UV irradiation) in *M. thermoacetica* membranes had little effect on the reduction of cytochrome b_{559} (equivalent to b_{561} in the present study) by CO (a physiological electron donor) and that the addition of oxidized menaquinone rapidly oxidized the reduced cytochrome b_{559} (19). In the *E. coli* cytochrome *bd* oxidase, the reduction of cytochrome *d* is coupled to b_{561}, which mediates transfer of electrons between quinol and cytochrome *d* (32). In *M. thermoacetica* membranes, the present results suggest that either electrons are transferred directly from quinols to cytochrome *d*, bypassing cytochrome b_{561}, or cytochrome *d* rapidly reoxidizes the quinol-reduced b_{561}.

TABLE 2. Quinol-dependent O_2 uptake rates by *M. thermoacetica* cytochrome *bd* oxidase preparations[a]

Preparation	Uptake rate (nmol · min^{-1} · mg of $protein^{-1}$)			
	Duroquinol[b]		Ubiquinol-1[b]	
	None	CN^-[c]	None	CN^-[c]
Membranes	7.2	2.2	5.5	1.4
DM extracts	15.0	3.7	12.0	3.0
Cytochrome *bd* oxidase[d]	25.0	3.7	18.2	2.8

[a] Measurements were carried out in 2-ml reaction mixtures containing membranes, DM extracts (100 μg of protein each), or cytochrome *bd* oxidase (50 μg of protein) at room temperature as described in Materials and Methods. Listed values are averages of at least three separate experiments with standard deviations of 0 to 10%.
[b] Quinol concentration was 100 μM.
[c] Cyanide concentration was 1 mM.
[d] Partially purified from the hydroxyapatite column as described in Materials and Methods.

Quinol oxidase activity of *M. thermoacetica* cytochrome *bd* oxidase was determined by measuring O_2 uptake by membranes, DM extracts, and an enriched cytochrome *bd* oxidase fraction partially purified from a hydroxyapatite column with DQH_2 or QH_2 as the electron donor. The data in Table 2 show that the rates of O_2 uptake by membranes, DM extracts, and the partially purified cytochrome *bd* oxidase were 31, 25, and 37% higher, respectively, with DQH_2 than with QH_2. Significantly higher O_2 uptake activity of cytochrome *bd* oxidase with DQH_2 than QH_2 as a substrate was also reported for the *D. gigas* cytochrome *bd* oxidase (50). The quinol-dependent O_2 uptake in all three preparations was strongly inhibited (up to

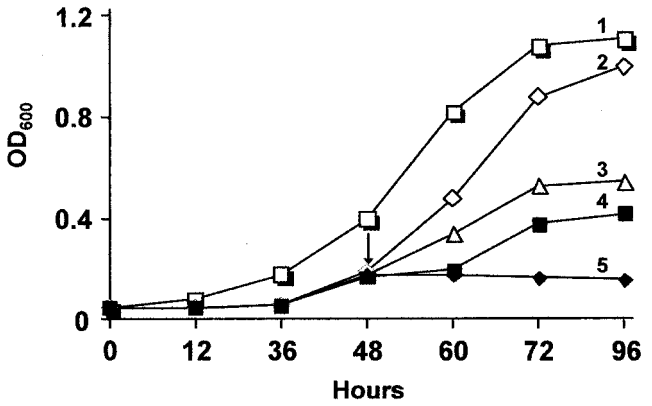

FIG. 9. Effect of oxidative stress on growth of *M. thermoacetica*. Cultures were grown on methanol (200 mM) in Drake's medium (65) (80 ml) under 100% CO_2 in anoxic bottles (125-ml capacity) in the presence (trace 1) or absence (traces 2 through 5) of reducing agent (cysteine + H_2S). After 48 h, either cysteine (2.8 mM) or azide (2 mM) was injected (shown by arrow) into replicate cultures grown in the absence of reducing agent followed immediately by injection of air into the headspace to give a final dioxygen concentration of 1.5 vol% (based on 21 vol% dioxygen in air). The cultures were shaken vigorously for 20 s (to equilibrate dioxygen) and then incubated at 58°C. Traces: 2, cysteine + dioxygen; 3, no treatment; 4, dioxygen only; 5, azide (2 mM) + dioxygen. At the times indicated, 1-ml aliquots of the cultures were withdrawn via syringe and centrifuged, and the cell pellets were suspended in equal volumes of 50 mM Tris-HCl, pH 8.0, prior to measurement of the OD_{600}. The results are average of three replicate experiments with standard deviations between 0 and 8%.

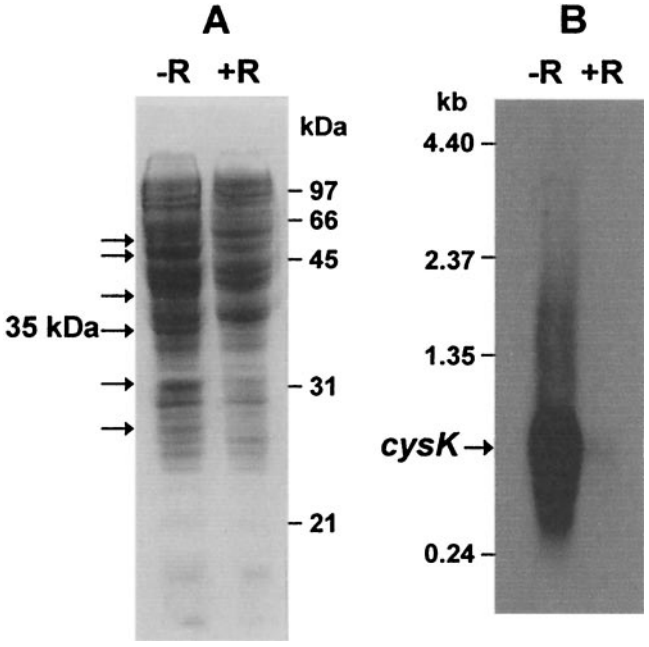

FIG. 10. (A) SDS-PAGE (12% acrylamide in resolving gel) of cytosolic extracts (40 μg of protein) of *M. thermoacetica* grown in the presence (+R) or absence (−R) of reducing agent. Proteins overexpressed in the absence of reducing agent were indicated by arrows. (B) Northern blot of total RNA (5 μg in each lane) isolated from *M. thermoacetica* grown on methanol in the presence (+R) or absence (−R) of reducing agent after hybridization with DIG-labeled DNA fragments amplified from *cysK*.

75%) by cyanide (1 mM), as also reported in other bacteria (30, 38, 46).

Oxidative stress protection by cytochrome *bd* oxidase and cysteine. Growth of *M. thermoacetica* in the absence or presence of cysteine, dioxygen, and azide (a cytochrome *bd* oxidase inhibitor) was investigated. Cultures were grown in anoxic bottles with the indicator dye resazurin (0.001% [wt/vol]), which turns pink in the presence of traces of dioxygen. Figure 9 shows that the maximum growth (OD_{600}) of the cultures nearly doubled in the presence compared to the absence of reducing agent (Fig. 9). After 48 h of growth, air was injected into the headspace of the culture bottles so as to reach an initial dioxygen concentration of 1.5 vol% (unreduced cultures failed to grow at headspace dioxygen concentrations above 1.5%). This addition of dioxygen had no effect on cultures grown in the presence of reducing agent (not shown), but those grown in the absence of reducing agent grew only after a lag of 12 h. During this lag period, the resazurin gradually changed from pink to colorless, indicating consumption of dioxygen. Similar results have been reported for other acetogenic bacteria (7). Unreduced cultures treated with cysteine prior to the exposure to dioxygen grew without any lag (and the resazurin quickly changed from pink to colorless) and had a maximum growth (OD_{600}) comparable to that of reduced cultures (Fig. 9), indicating that cysteine protected the cultures from growth inhibition by oxidative stress. On the other hand, addition of sodium azide (2 mM) to unreduced cultures prior to dioxygen addition inhibited growth (and the resazurin color remained pink throughout), indicating that the inhibition of cytochrome *bd* oxidase by azide diminished the ability of the cultures to reduce dioxygen. These results suggest protective roles of both cysteine and cytochrome *bd* oxidase against dioxygen and oxidative stress in *M. thermoacetica*.

Induction of a cysteine synthase in *M. thermoacetica*. The protein profile of cell extracts of *M. thermoacetica* cultures grown in either the presence or absence of reducing agent is shown in Fig. 10. Several proteins (marked by arrows) were expressed at higher levels in unreduced cultures (−R) than in reduced cultures (+R), including a 35-kDa protein (Fig. 10A). The N-terminal sequence of this 35-kDa protein was determined to be A/MKIARDVTQLIGQT, which is identical to the corresponding amino acid sequence of a predicted 306-residue protein encoded by an open reading frame of 918 bp in the nucleotide sequence of a small contig (contig 132) in the *M. thermoacetica* genome sequence. The calculated molar mass of this putative protein, 32,619 Da, is in close agreement with the SDS-PAGE-estimated value of 35 kDa, and its predicted amino acid sequence has high homologies (between 68 and 55% identities) to cysteine synthase K from *B. subtilis* (accession no. S66103), *M. tuberculosis* (accession no. G70660), and *Clostridium acetobutylicum* (accession no. E97175). We, therefore, refer to this 35-kDa *M. thermoacetica* protein as CysK.

In order to investigate the transcription of this *cysK*, Northern blots containing total RNA from *M. thermoacetica* were hybridized with DIG-labeled PCR products amplified from the *cysK* of contig 132. A strong hybridization band corresponding to an approximate size of 1 kb (Fig. 10B) was found on blots containing RNA from cells grown in medium lacking reducing agent (−Red), whereas little or no such band was found in the

lane from cells grown in medium containing reducing agent (+Red). The size of the band is in close agreement with the size of the putative *cysK*, 918 bp. A putative promoter sequence, 5′-CTTGACA-N_{26}-AATAAT-N_{23}-AGGAG-3′, resembling the *E. coli* consensus s^{70} promoter (5′-CTTGACa-$N_{15–21}$-TATAaT-AGGAG-3′) (33) was found upstream of the *cysK* start codon. C*ysK* transcription was, thus, apparently strongly repressed by the exogenous reducing agent.

DISCUSSION

Cytochrome *bd* oxidase has not been previously reported from an acetogenic or, to our knowledge, any gram-positive strictly anaerobic bacterium. Based on subunit composition and spectroscopic and biochemical properties, the *M. thermoacetica* cytochrome *bd* oxidase closely resembles the corresponding enzyme from other bacteria, including both aerobes and anaerobes (30, 36, 55). The *M. thermoacetica cyd* operon consists of four genes encoding CydA, CydB, CydD and CydC, which we verified are transcribed in a single polycistronic message. Several membrane-spanning conserved domains, including nine transmembrane helices of CydA and the ligands to the iron of the three different hemes were found to be conserved in the *M. thermoacetica* cytochrome *bd* oxidase, which reinforces the universal lineage of this enzyme among the bacteria (2).

Recently, hydrogen (a substrate in the Wood-Ljungdahl pathway) was shown to support dioxygen uptake in several acetogens (7). Results described herein show that dioxygen uptake by *M. thermoacetica* cell suspensions at room temperature were stimulated by CO or methanol and strongly inhibited by azide and cyanide, which are specific inhibitors of cytochrome *bd* oxidase. Apparently, the majority of the O_2 uptake (~70% based on cyanide inhibition) by *M. thermoacetica* cell suspensions is via cytochrome *bd* oxidase. Based on inhibition studies, dioxygen uptake (presumably reduction) by *M. thermoacetica* cytochrome *bd* oxidase is coupled to the membrane electron transport chain, as reported in other bacteria (59). Rates of dioxygen uptake and the level of *cyd* transcript were both higher in *M. thermoacetica* cells grown in nonreducing medium, further supporting the involvement of cytochrome *bd* oxidase in O_2 uptake and tolerance. Finally, growth of *M. thermoacetica* under nonreducing conditions with trace dioxygen was inhibited by azide, a known inhibitor of cytochrome *bd* oxidase.

Homologs of several proteins implicated in oxidative stress protection are encoded in the *M. thermoacetica* genome (http://genome.jgi-psf.org/draft_microbes/mooth/mooth.draft.html), including "anaerobic type" superoxide reductase, rubrerythrin, type A flavoprotein and rubredoxin, some of which have been characterized (20, 66). The *M. thermoacetica* genome also encodes "aerobic type" Fe/Mn superoxide dismutase (SOD) and Mn catalase, which have not been previously reported in *M. thermoacetica*. Curiously, while Karnholz et al. detected NADH oxidase activity, they did not detect SOD or catalase activities (at either 50°C or room temperature) in extracts of *M. thermoacetica* cells that had been exposed to low levels of dioxygen in growth medium lacking reducing agent (39).

There is now increasing evidence that cytochrome *bd* oxidase plays a crucial role in the survival and oxidative stress protection of a wide variety of facultative and anaerobic bacteria, at least under microaerobic growth conditions (2, 23, 51, 74). Mutation or deletion of the genes encoding cytochrome *bd* oxidase has been shown to increase sensitivity to oxidative stress in *E. coli* (51, 74) and *A. vinelandii* (23). The ability of cytochrome *bd* oxidases to reduce dioxygen directly to water may minimize the generation of toxic reactive oxygen species and also protect crucial metabolic enzymes in their dioxygen-sensitive reduced states (67). Amino acid sequence comparisons of CydAs from bacteria and archaea indicated that cytochrome *bd* oxidase is present in some of the most ancient bacteria and, therefore, that sufficient atmospheric dioxygen may have been present to support dioxygenic respiration even prior to the emergence of organic photosynthesis (2). It is unclear whether cytochrome *bd* oxidase can generate energy from the reduction of dioxygen in *M. thermoacetica*. However, the CO- and methanol-dependent reduction of O_2 by cytochrome *bd* oxidase in *M. thermoacetica* demonstrated in this work suggests coupling of the *bd* oxidase dioxygen reduction pathway to the membrane electron transport chain of the Wood-Ljungdahl pathway.

The growth of *M. thermoacetica* in nonreducing medium also led to increased levels of several other proteins, including a 35-kDa protein that we identified as CysK, a cysteine synthase. Expression of *cysK* has been reported to be a general response to stress in bacteria and plants (5, 57, 71, 73). In bacteria, cysteine is synthesized from *O*-acetylserine and H_2S catalyzed by *O*-acetylserine sulfhydrolase (CysK) while *O*-acetylserine is produced from serine and acetyl-CoA catalyzed by serine acetyltransferase (CysE) (12, 29). In the *M. thermoacetica* genome, a CysE homolog is encoded by gene 521 on contig 282. The *M. thermoacetica* genome also encodes a second CysK homolog (32,722 Da), encoded by gene 55 on contig 256, which shares 66% identical residues with the CysK found to be induced under nonreducing growth conditions in this work. The presence of two CysK homologues has also been reported in the gram-positive bacterium *Lactococcus lactis* (29). Stimulation of growth of unreduced *M. thermoacetica* cultures by exogenous cysteine and induction of *cysK* transcription and expression in the absence of added cysteine and H_2S in the growth medium suggest that CysK could also play an important role in oxidative stress protection in this bacterium. In many microorganisms, cysteine-containing molecules, (e.g., glutathione and thioredoxin) play major roles in maintaining an intracellular reducing environment and in protection against oxidative and other stress conditions (9, 22, 52). Cell extracts of *M. thermoacetica* can produce cysteine from cystine by enzymatic reduction with molecular hydrogen as electron donor (44). Results described herein thus provide strong evidence that both cytochrome *bd* oxidase and cysteine (and, by inference, CysK) play significant roles in dioxygen tolerance and oxidative stress protection of *M. thermoacetica* by scavenging dioxygen and/or maintaining a low intracellular reduction potential, both of which are known to be essential for growth of many anaerobic bacteria (1, 72).

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