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Adaptive Responses to Oxygen Stress in Obligatory Anaerobes *Clostridium acetobutylicum* and *Clostridium aminovalericum*

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***Clostridium acetobutylicum* and *Clostridium aminovalericum*, both obligatory anaerobes, grow normally after growth conditions are changed from anoxic to microoxic, where the cells consume oxygen proficiently. In *C. aminovalericum*, a gene encoding a previously characterized H_2O-forming NADH oxidase, designated *noxA*, was cloned and sequenced. The expression of *noxA* was strongly upregulated within 10 min after the growth conditions were altered to a microoxic state, indicating that *C. aminovalericum* NoxA is involved in oxygen metabolism. In *C. acetobutylicum*, genes suggested to be involved in oxygen metabolism and genes for reactive oxygen species (ROS) scavenging were chosen from the genome database. Although no clear orthologue of *C. aminovalericum* NoxA was found, Northern blot analysis identified many O_2-responsive genes (e.g., a gene cluster [CAC2448 to CAC2452] encoding an NADH rubredoxin oxidoreductase–A-type flavoprotein–desulfoferrodoxin homologue–MerR family-like protein–flavodoxin, an operon [CAC1547 to CAC1549] encoding a thioredoxin-thioredoxin reductase-glutathione peroxidase-like protein, an operon [CAC1570 and CAC1571] encoding two glutathione peroxidase-like proteins, and genes encoding thiol peroxidase, bacterioferritin comigratory proteins, and superoxide dismutase) whose expression was quickly and synchronously upregulated within 10 min after flushing with 5% O_2. The corresponding enzyme activities, such as NAD(P)H-dependent peroxide (H_2O_2 and alkyl hydroperoxides) reductase, were highly induced, indicating that microoxic growth of *C. acetobutylicum* is associated with the expression of a number of genes for oxygen metabolism and ROS scavenging.**

Bacteria belonging to the genus *Clostridium* are classified as obligatory anaerobes (26, 62) and are widely used in the field of solvent fermentation, biodegradation, and microbial energy production. Oxygen has a crucial effect on the growth of clostridia, but the mechanisms of growth inhibition, as well as the existence of O_2 metabolic systems, remain unknown. Some hypotheses to explain aerobic growth inhibition in anaerobes were proposed, such as the possibility that oxygen attacks oxygen-sensitive enzymes causing metabolic cessation or that anaerobes lack the ability to decompose active oxygen species, such as catalase, which cause irreversible oxidative damage to DNA and lipid molecules (2, 27, 49, 63, 67). O'Brien and Morris proposed that NAD(P)H oxidation systems react with oxygen to cause oxidation of the electron donor, i.e., NAD(P)H, which is required for the central pathway for anaerobic metabolism; this then leads to the eventual inability of clostridia to maintain their internal redox balance (51, 55). However, many questions remain about the mechanisms of aerobic growth inhibition in clostridia (50).

Most *Clostridium* species do not form colonies in the presence of 1% oxygen (2, 62); however, they can accept microoxic conditions when grown in liquid medium (32–35, 39, 51, 55). Based on physiological examination, clostridia possess systems to metabolize O_2, and oxygen itself does not have an irreversible effect on growth. *Clostridium butyricum*, a type species of the genus *Clostridium*, has the ability to resume growth after oxygen consumption without any lasting damage (33). Küsel et al. reported that *Clostridium glycolicum*, an acetogenic bacterium, can grow in the presence of oxygen (under up to 6% headspace oxygen in static culture) with oxygen-consuming activities. Under these conditions, this acetogen switches to a fermentative metabolism that is not as sensitive to oxygen as acetogenesis (39). Karnholz et al. reported that *Clostridium magnum*, an acetogenic bacteria, can grow normally in nonreduced liquid culture medium in the presence of oxygen (1% headspace oxygen) with oxygen-consuming activity and with normal acetogenesis (32). *Clostridium acetobutylicum* and *Clostridium aminovalericum*, both typical *Clostridium* species, grow normally under continuous microoxic conditions by consuming oxygen (34, 35). These observations suggest that clostridia should possess some systems to metabolize O_2 as well as to scavenge active oxygen species derived from O_2 reduction; however, the existence of these systems has not been well investigated.

Based on the recent molecular-based approach to finding clostridial oxygen metabolic systems, some oxygen response genes have been identified. In the pathogen *Clostridium perfringens*, several genes (CPE0781, encoding a probable flavoprotein; CPE0782, encoding an alkyl hydrogen peroxide reductase; and CPE0782, encoding a superoxide dismutase [SOD]) respond to air at the transcriptional level (28). In *C. acetobutylicum*, novel

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oxygen response proteins (A-type flavoprotein and rubrerythrins) were identified by two-dimensional electrophoresis, and the transcripts encoding these proteins were found to be upregulated quickly and strongly within minutes after the cells were exposed to microoxic aeration (35). Although the above-mentioned enzyme activities and their roles in vivo have not been determined, it is obvious that clostridia possess systems to respond to O_2 at the transcriptional level.

In this study, we investigated the expression of genes required for oxygen metabolic systems using two species, *C. aminovalericum* and *C. acetobutylicum*. In *C. aminovalericum*, an H_2O-forming NADH oxidase was purified for the first time from a member of the *Clostridium* genus and was characterized as a kinetically functional oxidase in vivo (34). Expression studies of the *C. aminovalericum noxA* gene revealed that the enzyme is involved in oxygen metabolism. In *C. acetobutylicum*, many genes were identified as oxygen stress responsive for the first time in the genus *Clostridium*. The possible functions of these O_2-responsive gene products are discussed.

MATERIALS AND METHODS

Bacterial strains and media. *Clostridium aminovalericum* NRIC0223 (= DSM1283) and *Clostridium acetobutylicum* DSM792 (= ATCC 824) were used in this study. Growth conditions and aeration studies were described previously (34, 35). Briefly, anoxic cultures were achieved by flushing the medium with O_2-free nitrogen gas in a jar fermenter (10-liter jar fermenter, model MDL1000; Marubishi-Bioenge, Tokyo, Japan). Aeration was achieved by flushing with 3% O_2–97% N_2 mixed gas or with 5% O_2–95% N_2 mixed gas (a gas flow meter was used to fix the ratio of each gas) into the anaerobically growing strain at the mid-exponential phase.

Cloning of a *C. aminovalericum* H_2O-forming NADH oxidase. We previously determined the N-terminal 13 amino acids of *C. aminovalericum* NADH oxidase (34). To obtain genomic DNA encoding *C. aminovalericum* NADH oxidase, we performed long N-terminal sequencing again. The methods for sequencing were described previously. The N-terminal amino acid sequence (40 amino acids from the N terminus) was determined by the Edman degradation method with a peptide sequencer (model 92HT; Perkin-Elmer Applied Biosystems, Foster City, CA). We first isolated the partial genome region by using degenerated sense and antisense primers based on the N-terminal amino acid sequence. The degenerated sense primer (5′-ATGAARATHGTNGTNATHGG-3′ [where H is A, T, or C; N is A, C, G, or T; and R is A or G]) and antisense primers (5′-TCRAAD ATNGTDATYTCNGC-3′ and 5′-ATNGTDATYTCNGCYTCNGG [where D is A, G, or T and Y is C or T]) were used for the first PCR using *C. aminovalericum* genomic DNA as a template. Sequence analysis indicated that 96 bp of the DNA fragment obtained from the first PCR encoded the N-terminal part of the target protein. Using an in vitro cloning kit (Takara-bio, Tokyo, Japan), a DNA fragment of 489 bp including the 96-bp first PCR DNA fragment was obtained. The 489-bp fragment was used as a probe to screen a *C. aminovalericum* genomic library in the Lambda Zap bacteriophage. *C. aminovalericum* genomic DNA partially digested with the enzyme Sau3AI was used to construct a genomic DNA library. All steps for library construction and in vitro packing were performed according to the manufacturer's instructions (Lambda ZAP Express library construction kit and Gigapack III Gold Packing extracts; Stratagene). The probe was labeled by random priming using [^{32}P]dCTP as described previously by Sambrook et al. (59). The pBluescript plasmids containing chromosomal inserts were excised from positive plaques according to the manufacturer's instructions. One plasmid, pNoxA-10, was chosen for sequencing of the *noxA* genome region. Multiple sequence alignment was achieved with the program Clustal W.

Northern hybridization. All steps were performed according to methods described previously (34, 35). Briefly, total RNAs from cell lysates harvested at several time points of aeration were isolated using TRIzol (Invitrogen, CA) according to the instruction manual. Northern blotting was carried out by standard procedures (35). The RNA (15 μg) was electrophoresed in 1.0% agarose gels and blotted onto nylon membranes (Hybond N^+; Amersham, Japan). The membranes were probed with the PCR-amplified DNA fragment encoding the target region. The identity of the amplified DNA fragment was confirmed by size and nucleotide sequence. After the RNA was subjected to prehybridization at 60°C for 30 min, the ^{32}P-labeled DNA probe was hybridized to RNA on the membrane at 60°C for 12 h.

Enzyme assays. *C. aminovalericum* and *C. acetobutylicum* cells harvested at several time points of aeration were suspended in 50 mM potassium phosphate buffer (pH 7.0) and disrupted by treatment with a French pressure cell at 140 MPa. Cell extracts were obtained by removal of cell debris by centrifugation at 15,000 × *g* for 15 min.

The activities of NADH oxidase and NADPH oxidase were assayed spectrophotometrically in 1 ml of air-saturated 50 mM sodium phosphate buffer (pH 7.0) containing 0.15 mM NAD(P)H at 37°C. The reaction was started by the addition of enzyme solution, and the decrease in absorbance at 340 nm was monitored with a spectrophotometer (model U-160; Shimadzu, Kyoto, Japan). One unit of activity was defined as the amount of enzyme that catalyzes the oxidation of 1 μmol NAD(P)H per min.

The activities of NADH- and NADPH-dependent H_2O_2 or alkyl hydroperoxide reductase were assayed anaerobically in anaerobic glass cuvettes in 2 ml of Ar gas-saturated 50 mM sodium phosphate buffer (pH 7.0) containing 0.15 mM NAD(P)H at 37°C. The reaction was started by the addition of Ar gas-treated enzyme solution, and the decrease in absorbance at 340 nm after the addition of 1 mM H_2O_2, *t*-butyl hydroperoxide, or cumen hydroperoxide was monitored with a spectrophotometer (model U-160; Shimadzu, Kyoto, Japan). One unit of activity was defined as the amount of enzyme that catalyzes the oxidation of 1 μmol NAD(P)H per min.

Glutathione (GSH) peroxidase activities were measured indirectly by examining a coupled reaction with glutathione reductase (18). The principle is that the oxidized glutathione produced by the reduction of H_2O_2 by GSH peroxidase is recycled to reduced GSH in the presence of excess glutathione reductase and NADPH. The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0) containing 1 mM GSH plus 0.15 mM NADPH, 1 U glutathione reductase, and the enzyme, and the reaction was started by the addition of Ar gas-treated enzyme solution. The decrease in absorbance at 340 nm after the addition of 1 mM H_2O_2 was measured spectroscopically. One unit of activity was defined as the amount of enzyme that catalyzes the oxidation of 1 μmol NAD(P)H per min.

Superoxide dismutase activity was determined using the xanthine oxidase-cytochrome *c* system as described previously by McCord and Fridovich (49).

Nucleotide sequence accession number. The nucleotide sequence data reported in this study have been deposited in the DDBJ/EMBL/GenBank database under accession number AB219226.

RESULTS AND DISCUSSION

Cloning and characterization of the gene encoding H_2O-forming NADH oxidase from *C. aminovalericum*. *C. aminovalericum* NADH oxidase, which reduces O_2 to H_2O with a comparatively high affinity for O_2 (the K_m for oxygen is 61.9 μM), was purified and characterized (34). The sequence of the 40 amino acids from the N terminus was determined to be MKIVVIGCT HAGTAAVKTILKENPEAEITIFERNDNISFL. A 96-bp gene fragment encoding the N-terminal part of *C. aminovalericum* NADH oxidase was cloned using an oligonucleotide designed from the N-terminal sequence. Using the 96-bp nucleotide fragment, a genome fragment of approximately 4 kb was cloned and sequenced, and three open reading frames (ORFs) were identified as *orf1*, *noxA*, and *orf2* (Fig. 1A). The cloned gene for NADH oxidase was 1,347 bp long and encoded 448 amino acid residues. The primary structure of NADH oxidase deduced from *noxA* shows high sequence similarity to *E. faecalis* NADH oxidase (NCBI accession no. AAO81372; 66% identity). The proposed domains for flavin adenine dinucleotide binding and NADH binding and the site of a redox-active cysteine residue in *Enterococcus faecalis* NADH oxidase (Cys42) (58) are conserved in *C. aminovalericum* NoxA. By the genome database search for investigating the distribution of this protein in clostridia, *C. aminovalericum* NoxA homologues were identified in *Clostridium perfringens* (accession no. BAB80440; 29% identity) and *Clostridium tetani* (accession no. AAO35751; 28% identity). Both proteins conserved redox-active Cys42 and domains for flavin adenine dinucleotide bind-

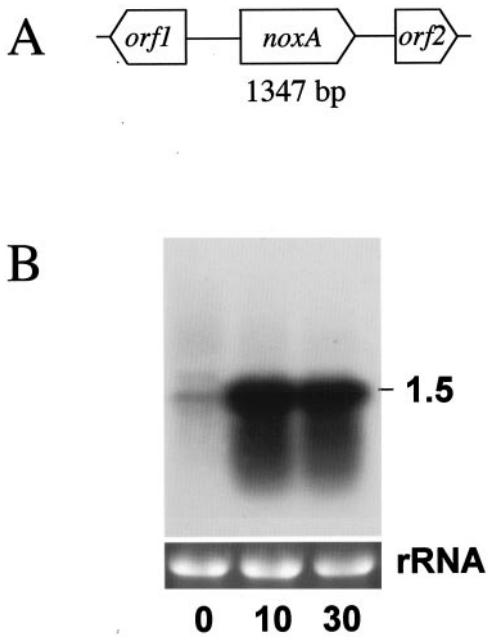

FIG. 1. Cloning and expression of the *noxA* gene of *C. aminovalericum*. (A) Genome structure around the *noxA* gene. (B) Northern blot of 15 μg of *C. aminovalericum* total RNA probes with *noxA* amplified by PCR. 0, just before the start of aeration with 3% O_2–97% N_2 at the mid-exponential phase; 10, after 10 min of 3% O_2–97% N_2 aeration; 30, after 30 min of 3% O_2–97% N_2 aeration. The estimated sizes of the observed transcripts are indicated on the right. Ethidium bromide staining of the rRNA genes for confirmation of equal loading of RNA samples is shown below the autoradiogram.

ing and NADH binding, suggesting that they function as NADH oxidase, but both proteins have not been characterized in terms of function.

ORF1, located 641 bp upstream from the start codon of *noxA*, encodes a protein of 211 amino acids, and proteins showing similarity are found in *Streptococcus mutans* (called hypothetical protein SMU.1470c [NCBI accession no. NP_721820]; 46% identity) and *Enterococcus faecium* (called uncharacterized conserved protein [accession no. ZP_00287298]; 46% identity). None of these proteins has been characterized in terms of function. ORF2, located 481 bp downstream from the terminal codon of *noxA*, encodes a protein of 156 amino acids, and similar proteins are found in *Enterococcus faecium* (called a putative transposase [accession no. AAF73101]; 78% identity) and *C. acetobutylicum* (called IS*605*/IS*200*-like transposase [accession no. AAK81457]; 73% identity).

Transcriptional expression of genes encoding NADH oxidase from *C. aminovalericum*. We assumed that the *C. aminovalericum* NADH oxidase would play a central role in the oxygen consumption observed in this bacterium (34). Actually, the activity of NADH oxidase in the cell extract was activated more than sixfold after 30 min of flushing with 3% O_2 (34). To determine whether the expression of the *noxA* gene corresponds with the elevation of enzyme activity, Northern analysis was performed (Fig. 1B). The results show that the *noxA* gene was monocistronically transcribed and strongly up-regulated by 10 min after the start of microoxic aeration. These results, as well as the enzyme kinetics data (34), indicate that NoxA is involved in the oxygen metabolic system of *C. aminovalericum*.

Examination of the *C. acetobutylicum* genome to identify the genes involved in oxygen metabolism and active oxygen scavenging. For further attempts to identify the genes that are required for oxygen metabolic systems in clostridia, we used *C. acetobutylicum*, a well-investigated solvent producer that grows microoxically by consuming oxygen, as in the case of *C. aminovalericum*. The whole genome sequence of *C. acetobutylicum* is available in public databases (54). By using key words such as oxygen metabolism, reactive oxygen species (ROS) scavengers, and the maintenance of the redox environment of a cell, we examined the *C. acetobutylicum* genome database for choosing genes including *C. aminovalericum* NoxA homologues. The genes chosen in this study are listed in Table 1. The functions of most of these proteins in *C. acetobutylicum* have not been characterized, and they are all presumed to be located in cytosol. The specific primers used to amplify the target genes are listed in Table 1. Biological reduction of oxygen typically occurs by means of terminal oxidases such as cytochrome oxidase. Interestingly, cytochrome *bd*-type oxidases have recently been discovered in strict anaerobes such as *Bacteroides* species (5), *Desulfovibrio gigas* (42), and *Moorella thermoacetica* (16) as a functional terminal oxidase. In the *C. acetobutylicum* genome, genes encoding cytochrome *bd*-type oxidase or other membrane-bound-type oxidases are not found.

O_2-responsive *nror* gene cluster. In *C. acetobutylicum*, the database search identified several genes that encode *C. aminovalericum* NoxA homologues; however, their identity scores were not high (the products of genes CAC0764, CAC1674, CAC3371, and CAC3408 show homology but less than 17% identity with NoxA). The *nror* gene (CAC2448) encodes a protein showing the closest similarity with *C. aminovalericum* NoxA (22% identity) in the *C. acetobutylicum* genome. This protein was formerly characterized as NADH-rubredoxin oxidoreductase (NROR) by Guedon and Petitdemange (24). Although the reactivity of *C. acetobutylicum* NROR with oxygen has not been determined, redox-active Cys42 is not conserved in *C. acetobutylicum* NROR, suggesting that NROR itself does not function as an NADH oxidase. *C. acetobutylicum* NROR reduces rubredoxin efficiently; however, its actual function in vivo has not been elucidated because the *rd* gene (accession no. CAC2778), which encodes rubredoxin, the expected electron acceptor from NROR, is located 340 kb apart from *nror* (24). Northern blot analysis showed that *nror* is transcribed tricistronically with other two genes (CAC2449, named *fprA2*, and CAC2450, named *dsr*), which encode an A-type flavoprotein homologue and a desulfoferrodoxin homologue, respectively (Fig. 2A). These genes are faintly expressed during anaerobic growth but are strongly upregulated within 10 min of the start of aeration (Fig. 2B). To our knowledge, there is no study on O_2-responsive *nror* in bacteria. Interestingly, the *dsr* gene is transcribed not only tricistronically with *nror* and *fprA2* genes but also monocistronically by its own promoter. By using the *dsr* region as a probe, tricistronic upregulation after 10 min was observed, and also, strong upregulation of *dsr* was detected after 30 min (Fig. 2C). These results suggested that two types of transcriptional regulators regulate the expression of the *dsr* gene. We are now analyzing the expression mechanism of the *dsr* gene.

TABLE 1. List of genes and PCR primers used in this study

Gene identification	Gene name	Primer sequence (5′–3′)
nror gene cluster		
CAC2448	*nror*	F, AGATGATTTATATGAAAAGCAC
		R, AATGTATTTATCTTCTTGTG
CAC2449	*fprA2*	F, AGTTCTAAATCCTAGTCTCC
		R, CTCAGATGGAACAAATAAAC
CAC2450	*dsr*	F, ATGAATAACGATTTATCAATTTAC
		R, TTATATATCTGCTTTCCATAGG
CAC2451	*orf2451*	F, GAGCTTAATATAATAGTTCC
		R, ACATTTATTTAATAGCAGCC
CAC2452	*fld1*	F, GTCGAGGAGGAATTATTATG
		R, TCTTCCTTACTAGGTGCCTC
Glutathione peroxidase operon 1		
CAC1547	*trxA1*	F, GGCAATGAAATGATACAAGAG
		R, TGTCCATAGAAATCCACTCC
CAC1548	*trxB1*[a]	
CAC1549	*gpx3*[a]	
Glutathione peroxidase operon 2		
CAC1570	*gpx1*	F, TTGGGAGGTACGATTATGTC
		R, TCTTCCATGCTTATATCTTC
CAC1571	*gpx2*[a]	
Thioredoxin-thioredoxin reductase operon		
CAC3082	*trxB2*	F, AGGAGAGCCTGTAATAGTTG
		R, TAAAGGAGCATCACAAGTGG
CAC3083	*trxA2*[a]	
BCP and TSA family proteins		
CAC0327	*bcp*	F, TTTAGAGGTGATATTTATGG
		R, TCCTATTGGCGTTTTCTACC
CAC3306	*tpx*	F, TAAACAGTATGCTATGAAAG
		R, GCTGCTTGTAACACTTCATC
Rubrerythrin homologue		
CAC2575	*rubY*	F, TATATGAAATCACTTAAAGG
		R, TGAGGATGAAGACATGCTGG
CAC3018	*rubZ*	F, GGAAGTATATTATTATGAGTG
		R, CATTGTTCTATATTCTCATC
SOD homologue		
CAC1363	*sodC*	F, TGAACACAGAGAGAACGAGG
		R, CTAACAGCTCTAATAACACC
CAC2567	*sodB*	F, AATTTTAATTCACTAGCTGG
		R, ATAGACATAAAGGGACATAC

[a] Genes whose expression levels were determined to be the same as that of a gene in a polycistronic unit.

Interestingly, *fprA2* encodes a protein showing homology to an oxygen-induced A-type flavoprotein (NCBI accession no. NP_347663) (encoded by CAC1027, named *fprA1*; 40% identity) identified by two-dimensional electrophoresis in our previous study (35). FprA homologues are distributed predominantly in anaerobes. *Desulfovibrio vulgaris* FprA (accession no. Q9F0J6) and *Moorella thermoacetica* FprA (accession no. Q9FDN7) were recently determined to function as nitric oxide reductases, but not as oxidases, because oxidase turnover causes irreversible inactivation of the enzymes (60, 61). *C. acetobutylicum* FprA2 shows 28% identity with *D. vulgaris* FprA and *M. thermoacetica* FprA; however, the numbers and positions of the conserved cysteine residues, which are important for iron binding and the redox state, do not correspond. Thus, there is a possibility that oxygen-responsive *C. acetobutylicum* FprA1 and FprA2 are involved in the oxygen metabolic system.

C. acetobutylicum Dsr shows homology to *Desulfovibrio vulgaris* desulfoferrodoxin (NCBI accession no. P20418; 43% identity) and *Desulfovibrio desulfuricans* desulfoferrodoxin (accession no. P22076; 42% identity) (7, 52). Desulfoferrodoxin is reported to catalyze the superoxide reductase (SOR) reaction (44, 45). The SOR reaction is believed to be advantageous to anaerobic bacteria, in comparison to SOD, because SOR produces no oxygen by reducing O_2^- (23, 29). In *C. acetobutylicum* Dsr, one Cys-X-Y-Cys and one Cys-Cys motif that are conserved in *Desulfovibrio* proteins and are responsible for the binding of redox-active center I [$Fe(SCys)_4$], which has a distorted tetrahedral sulfur coordination sphere and is presumably an electron transfer center (17), are not conserved. *C. acetobutylicum* Dsr contains a [$Fe(His)_4Cys$] site, called center II in desulfoferrodoxin family proteins (12, 68). Lombard et al. reported that center II is the O_2^--reactive center because it is rapidly oxidized by O_2^- (45). A BLAST search found one orthologue of *C. acetobutylicum* Dsr, which lacks center I, in the genome database of *Treponema pallidum* (named desulfoferrodoxin [accession no. AAC65791]; 36% identity). This protein has been well characterized and was reported to function as superoxide reductase (31, 46). These results suggested that *C. acetobutylicum* Dsr belongs to the same family of desulfoferrodoxins and functions as superoxide reductase. Based on the gene structure and the expression profile, we propose that the proteins encoded by the *nror* operon might function as novel multiple-enzyme complexes (NROR-FprA2-Dsr) in NAD(P)H-dependent oxygen metabolism or ROS scavenging.

There are two genes localized downstream of *dsr* (Fig. 2A), one of which encodes a methyltransferase homologue fused to a MerR-type transcriptional regulator homologue at the N-terminal part (CAC2451, named ORF2451). A BLAST search revealed no fusion proteins distributed among organisms. MerR-type transcriptional regulators have been found in a wide range of bacterial genera and have been shown to mediate environmental responses to stress including heavy metals, drugs, or oxidative stress (1, 6, 56, 65). The MerR-type family regulators conserve homologous N-terminal helix-turn-helix DNA-binding domains but different C-terminal domains, which enable them to bind a variety of coeffectors (inducers) (6). The N-terminal part of ORF2451 (60 amino acid residues from the N terminus) shows high similarity to the DNA-binding region of MerR-type regulatory proteins and conserves the helix-turn-helix domain (Fig. 3A). The C-terminal part (approximately 250 amino acid residues from the C terminus) shows similarity to some bacterial hypothetical proteins named menaquinone biosynthesis methyltransferase or *S*-adenosyl-L-methionine-dependent methyltransferase in genome projects (i.e., possible menaquinone biosynthesis methyltransferase in *Pyrococcus abyssi* [NCBI accession no. CAB49332]; 34% identity). These top-BLAST-hit proteins have not been characterized in terms of function. The C-terminal part of ORF2451 shows low similarity with the functionally identified *Escherichia coli C*-methyltransferase (named *ubiE* [accession no. M80749]; 13.5% identity and 50.6% similarity) (Fig. 3B). This protein is involved in the *S*-adenosyl-L-methionine-dependent *C*-methyl-

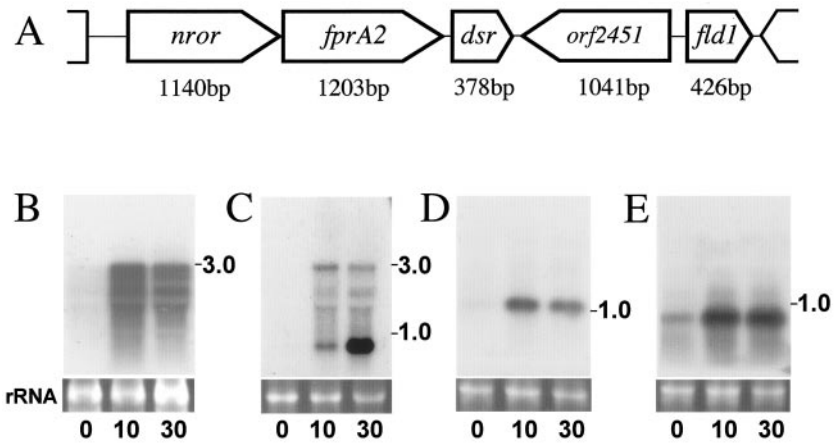

FIG. 2. (A) A gene cluster downstream of the *nror* gene (CAC2448) encoding NADH rubredoxin oxidoreductase. (B to E) Northern blots of 15 μg of *C. acetobutylicum* total RNA probed with (B) the *fprA2* gene, (C) the *dsr* gene, (D) the *orf2451* gene (CAC2451), or (E) the *fld1* gene amplified by PCR. The autoradiogram probed with the PCR-amplified *nror* gene showed a profile similar to that when *fprA2* was used as a probe (data not shown). 0, just before the start of aeration with 5% O_2–95% N_2 at the mid-exponential phase; 10, after 10 min of 5% O_2–95% N_2 aeration; 30, after 30 min of 5% O_2–95% N_2 aeration. The estimated sizes of the observed transcripts are indicated on the right. Ethidium bromide staining of the rRNA genes for confirmation of equal loading of RNA samples is shown below the autoradiogram.

transferase step of both ubiquinone and menaquinone biosynthesis (40). Thus, ORF2451 may be a novel MerR-type O_2-responsive transcriptional regulator with methyltransferase activity.

The gene named *fld1* (NCBI accession no. CAC2452) encodes a flavodoxin homologue. A BLAST search revealed highly homologous proteins distributed among *Clostridium* species. Flavodoxin is a flavin mononucleotide protein that replaces bacterial ferredoxin in hydrogen evolution and nitrogen fixation in vitro (37, 38). Flavodoxin is obtained from

A

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                    H   T   H
BmrR     --MKESYYSIGEVSKLANVSIKALRYYDKIDLFKPAYVDPDTSYRYYTDSQLIHLDLIKSLKYIGTPLEEMKKAQDLEM 77
MtaN     -----MKYQVKQVAEISGVSIRTLHHYDNIELLNPSALT-DAGYRLYSDADLERLQQILFARQVGFNLEESGELVNLFN 73
MerR     MENNLENLTIGVFAKAAGVNVETIRFYQRKGLLLEPDKP-YGSIRRYGEADVTRVRFVKSAQRLGFSLDEIAELLRLED 78

ORF2451  -------MNTKKVCEKLKISPKALRLYEELNIIVPN-RD-ENNYRNYSEDDLFKLRQVVVLKSIGIKLKSIKDILDKRK 70
CueR     -------MNISDVAKITGLTSKAIRFYEEKGLVTPPMRS-ENGYRTYTQQHLNELTLLRQFKEIGFRLDEIKEMLDHPN 69
                ** ..*.:   ::.**:*:*** .::.*  *. **.**.*:::.* :*  :  :*.**::*..**::**: :
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B

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ORF2451 100 ---LREGINTGLNNDISYEKLFDKIDECVYESEKNINSWIDKWEFDTWAKNYDDYVYNNKDSLGLFESYDLVLENV 173
E UbiE    1 MVDKSQETTHFGFQTVAKEQKADMVAHVFHSVASKYDVMNDLMSFG----IHRLWKRFTIDCSGVRR--GQTVLDL  70
                :  .    : :: *:  * : . .:.  .: :   *  .*.      :  :   .*. *: .  . .: ::

ORF2451    AKKIKGMNAKKVIDFGCGTANIVKKLSSSIEYTGVDQSIEMLIRARTKNKAINLRIGNFLDKPFAEEEFDAVISSFA 250
E UbiE     AGGTGDLTAKFSRLVGETGKVVLADINESMLKMGREK-----LRNIGVIGNVEYVQANAEALPFPDNTFDCITISFG 142
           *    .:.**    .*     :: .:..*:   * ::     :*       ::   .*    **.:: **.:  **.

ORF2451    FHHLNCYEKKRAIRYLLKYIKKNGKVIIADLMFLSKEEKMKKRQQ------------FIDNEEKDKWDTIEDEYYTD 315
E UbiE     LR--NVTDKDKALRSMYRVLKPGGRLLVLEFSKPIIEPLSKAYDAYSFHVLPRIGSLVANDADSYRYLAESIRMHPD 217
           ::  *  :*.:*:* : : :* .*:::: ::     *   *  :             . :: :. :: : . . :.*

ORF2451    IESLKAYIEDSGLKIKFEHIVNYTFIVEITA--- 346
E UbiE     QDTLKAMMQDAGFESVDYYNLTAGVVALHRGYKF 251
            ::*** ::*:*::    : :.  .:.   .
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FIG. 3. Amino acid sequence alignments. (A) Alignment of ORF2451 with the N-terminal DNA-binding domains of functionally characterized MerR family proteins. Proteins and their corresponding NCBI accession numbers are as follows: *B. subtilis* BmrR (accession no. CAB56371) (1), *B. subtilis* MtaN (accession no. CAB15677) (4), *P. aeruginosa* MerR (accession no. CAA83897) (56), and *E. coli* CueR (accession no. AAC73589) (57). H T H, DNA-binding helix-turn-helix motif. By comparison of ORF2451 and *E. coli* CueR, identical residues are asterisked, and similar residues are dotted (37% identity and 58% similarity). (B) Comparison of the amino acid sequence of the ORF2451 C-terminal region and the functionally characterized *E. coli* UbiE (E UbiE) (accession no. AAA67628) (40). Identical residues are asterisked, and similar residues are dotted.

Clostridium pasteurianum grown in iron-deficient medium (37). It is suggested that flavodoxin replaces ferredoxin in vivo since iron-deficient cells contain very little ferredoxin. These two genes are localized in tandem, but the *orf2451* gene (CAC2451) is in an opposite direction. Northern blot analysis revealed that the expression of both genes is up-regulated by 10 min after the start of aeration (Fig. 2C and D). In *Escherichia coli*, flavodoxin is reported to belong to the *soxRS* oxidative stress regulon, but the role of flavodoxin in oxidative stress is unknown (20).

Oxygen-responsive glutathione peroxidase, thioredoxin, and thioredoxin reductase. The genes that encode glutathione peroxidase homologues are located in different parts of the genome; two of them are localized tandemly as a dicistronic unit (CAC1570 [named *gpx1*] and CAC1571 [named *gpx2*]) (Fig. 4A). *gpx3* (CAC1549) is in the same polycistronic unit as two other genes, *trxB1* and *trxA1*, which encode a thioredoxin reductase homologue and a thioredoxin homologue, respectively (Fig. 4B). *gpx1* encodes a protein with 159 amino acid residues, and *gpx2* and *gpx3* encode proteins with 181 amino acid residues. Gpx1 and Gpx2 show 51% identity, and Gpx2 and Gpx3 show 55% identity. Gpx1 shows 55% identity with the functionally identified *Synechocystis* Gpx1 (19). Among bacterial genome data, it is difficult to find a gene cluster in which *gpx1* and *gpx2* are located in tandem. Northern analysis indicated that both *gpx1* and *gpx2* are dicistronically transcribed and strongly upregulated within 10 min after O_2 flushing (Fig. 4D). *gpx3* is also transcribed tricistronically and is strongly upregulated within 10 min after O_2 flushing (Fig. 4E). Gpx3 may function in a multiple-enzyme complex with thioredoxin and thioredoxin reductase for ROS scavenging.

A gene encoding a homologue of oxygen-responsive TrxB1 is found in the *C. acetobutylicum* genome. The gene CAC3082 encodes a thioredoxin reductase homologue named TrxB2 which shows 59% identity with TrxB1. *trxB2* is located in tandem with a gene encoding a thioredoxin homologue, named *trxA2* (CAC3083) (Fig. 4C). TrxA2 shows 41% identity with the above-mentioned oxygen-responsive TrxA1. Northern blot analysis indicated that *trxA2-trxB2* is dicistronically transcribed and expressed constitutively (Fig. 4F).

Rubrerythrin homologues. In *C. acetobutylicum*, tandemly linked rubrerythrins (NCBI accession no. NP_350180 and NP_350181) were identified as O_2-induced proteins, and the genes encoding these proteins are upregulated within 10 min after flushing with 5% O_2 (35). These genes are regulated by heat shock and H_2O_2 treatment (48). In the *C. acetobutylicum* genome, genes encoding rubrerythrin homologues are found as listed in Table 1. Unlike the two rubrerythrin homologues with conserved Fe-binding clusters at their N termini, the remaining two rubrerythrin homologues (RubY, encoded by a gene named *rubY* [CAC2575], and RubZ, encoded by a gene named *rubZ* [CAC3018]) have the conserved Fe-binding cluster in their C-terminal regions. This type of rubrerythrin is well characterized and reported to function as SOD or cytoplasmic peroxidase (13, 15, 21, 41, 47, 64). *rubY* was found to be moderately up-regulated within 10 min after O_2 flushing, but *rubZ* did not show strong up-regulation within 30 min after O_2 flushing (Fig. 5A and B). May et al. reported that the expression of *rubY* is not influenced by air stress (48). This result may be the reason that air stress

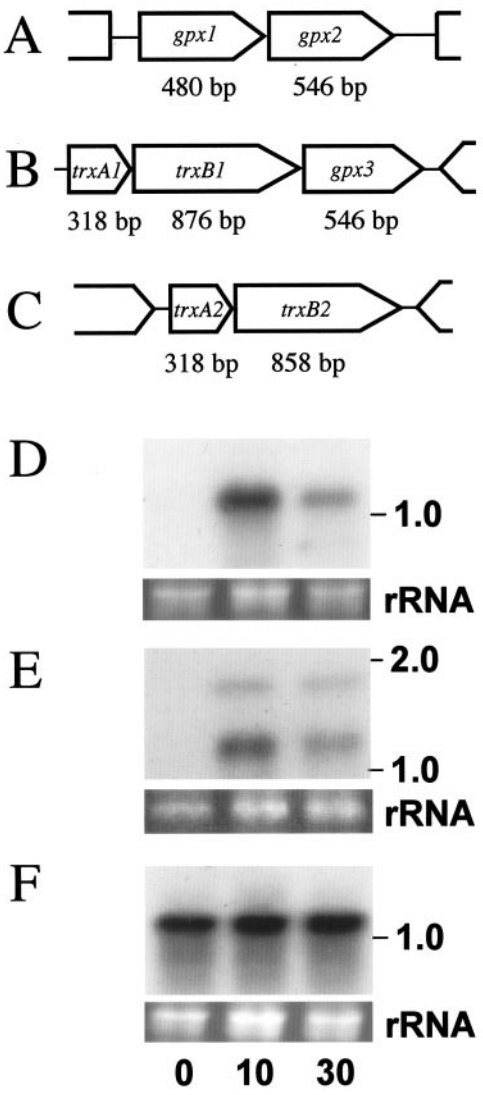

FIG. 4. Gene clusters of (A) two glutathione peroxidase-like proteins, (B) thioredoxin-, thioredoxin reductase- and glutathione peroxidase-like proteins, and (C) thioredoxin-thioredoxin reductase. (D to F) Northern blots of 15 μg of *C. acetobutylicum* total RNA probed with (D) the *gpx1* gene, (E) the *trxA1* gene, or (F) the *trxB2* gene amplified by PCR. 0, just before the start of aeration with 5% O_2–95% N_2 at the mid-exponential phase; 10, after 10 min of 5% O_2–95% N_2 aeration; 30, after 30 min of 5% O_2–95% N_2 aeration. The estimated sizes of the observed transcripts are indicated on the right. Ethidium bromide staining of the rRNA genes for confirmation of equal loading of RNA samples is shown below the autoradiogram.

(20% O_2) makes the medium highly oxic, which causes the cells to stop growing.

AhpC/TSA/BCP family proteins. Genes encoding thiol peroxidase (Tpx) family proteins were found in the *C. acetobutylicum* genome, suggesting that *C. acetobutylicum* possesses those antioxidative peroxiredoxins. Thiol peroxidases are mainly classified into three different clusters, the alkyl hydroperoxidase component (AhpC), bacterioferritin comigratory proteins (BCP), and thiol-specific antioxidant protein (TSA) (9, 25, 30). These thiol peroxidases reduce hydroperoxides with the use of other electron donor proteins (8, 30, 53). Genes encoding a BCP homologue

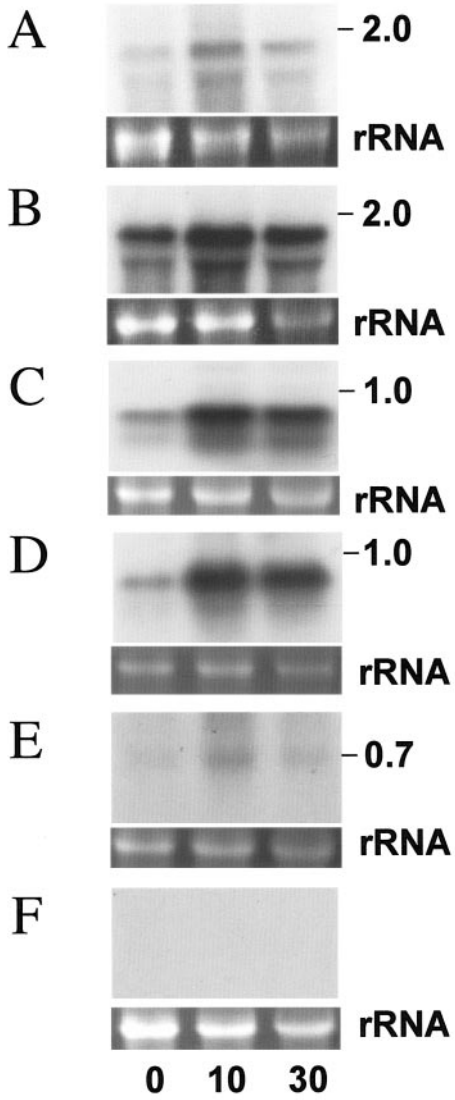

FIG. 5. Northern blots of 15 μg of *C. acetobutylicum* total RNA probed with (A) the *rubY* gene, (B) the *rubZ* gene, (C) the *bcp* gene, (D) the *tpx* gene, (E) the *sodC* gene, and (F) the *sodB* gene amplified by PCR. 0, just before the start of aeration with 5% O_2–95% N_2 at the mid-exponential phase; 10, after 10 min of 5% O_2–95% N_2 aeration; 30, after 30 min of 5% O_2–95% N_2 aeration. The estimated sizes of the observed transcripts are indicated on the right. Ethidium bromide staining of the rRNA genes for confirmation of equal loading of RNA samples is shown below the autoradiogram.

(CAC0327, named *bcp*) and a TSA homologue (CAC3306, named *tpx*), but no clear orthologue of AhpC, are found in the *C. acetobutylicum* genome. Tpx shows 38% identity with the functionally characterized *E. coli* Tpx (accession no. AAC45284). *C. acetobutylicum* Tpx has conserved Cys58 and Cys92 (corresponding to redox-active Cys61 and Cys95 in *E. coli* Tpx) but lacks Cys85, the deletion of which has a slight effect on the reaction rate of *E. coli* Tpx (3). Bcp shows 37% identity to the functionally characterized *E. coli* BCP (accession no. AAC75533), and the three cysteine residues (corresponding to Cys45, Cys50, and Cys99 in *E. coli* BCP) are well conserved (30). Northern blot analysis indicated that both genes are transcribed monocistronically and are highly expressed within 10 min after 5% O_2 flushing (Fig. 5C and D). To our knowledge, there is no study on the transcriptional response of *bcp* to oxidative stress in bacteria. In *E. coli*, expression of the gene encoding Tpx did not respond to oxidative stress (9).

SOD. Superoxide dismutase is well characterized in many organisms as a scavenger of the superoxide anions that arise from the one-electron reduction of oxygen (49). In aerobic organisms, this enzyme is essential for ROS scavenging; however, its role is not well characterized in clostridia. In *C. acetobutylicum*, the genes that encode an Fe/Mn-type SOD homologue (CAC2567, named *sodB*) and a Cu/Zn-type SOD homologue (CAC1363, named *sodC*) are located in different parts of the genome. SodB shows 31% identity with functionally characterized *Aquifex pyrophilus* Fe-SOD (accession no. 1COJA) (43). SodB does not show significant identity (24% in overlapping regions) with O_2-responsive *C. perfringens* CPE1236. SodC shows 33% identity with the functionally characterized chloroplast-localized Cu/Zn SOD of *Arabidopsis thaliana* (accession no. O78310) (36). Upon Northern blot analysis, *sodC* was found to be up-regulated within 10 min of O_2 flushing, but no expression of *sodB* was detected (Fig. 5E and F).

Enzyme activities for oxygen and active oxygen scavenging. In *C. acetobutylicum*, many genes that encode putative functional proteins related to oxygen metabolisms and ROS scavenging are up-regulated under microoxic growth conditions. These results suggest that many enzymes are required for normal growth under microoxic conditions. Enzyme activities after 30 min of flushing with O_2 are shown in Table 2.

The rate of activation of NAD(P)H oxidase activity was low compared to that of *C. aminovalericum*. This may be due to the lack of H_2O-forming NADH oxidase in *C. acetobutylicum*. Interestingly, the activities of NADH- and NADPH-dependent H_2O_2 reductase are dramatically increased after aeration. In addition, NADH- and NADPH-dependent alkyl hydroperoxide reductase activities (determined as *t*-butyl hydroperoxide and cumene hydroperoxide reductase activities) are also dramatically increased. In cyanobacteria, the glutathione peroxidase homologues Slr1171 and Slr1992 do not exhibit glutathione peroxidase activities but show NADPH-dependent alkyl

TABLE 2. Enzyme activities for oxygen and ROS scavenging in *C. acetobutylicum* after 30 min of 5% O_2–95% N_2 aeration

Enzyme	Activity[b] (mU/mg protein)		Induction (fold)
	0 min	30 min	
NADH oxidase	35.9 ± 1.3	74.2 ± 0.4	2.1
NADPH oxidase	6.93 ± 0.34	14.7 ± 0.65	2.1
NADH peroxide reductase			
H_2O_2	0.20 ± 0.08	23.1 ± 1.30	116
t-Butyl hydroperoxide	0.23 ± 0.12	4.75 ± 0.53	20.7
Cumene hydroperoxide	0.09 ± 0.03	4.40 ± 0.17	48.9
NADPH peroxide reductase			
H_2O_2	ND[b] (<0.1)	10.7 ± 0.20	>100
t-Butyl hydroperoxide	0.47 ± 0.10	4.24 ± 0.71	9.0
Cumene hydroperoxide	0.29 ± 0.18	3.71 ± 0.18	12.8
Glutathione peroxidase	0.87 ± 0.17	0.26 ± 0.09	0.3
SOD[a]	0.75 ± 0.29	0.97 ± 0.35	1.3

[a] U/mg protein.
[b] ND, not detected.

hydroperoxide reductase activities (19). In *C. acetobutylicum*, three genes encoding homologues of glutathione peroxidase that show homology to *Synechocystis* glutathione peroxidases are strongly up-regulated by aeration, but the corresponding glutathione peroxidase activity was not activated, suggesting that the NADPH alkyl hydroperoxide reductase activity might be carried out by these glutathione peroxidase-like proteins. In addition, genes encoding BCP and TSA are strongly up-regulated in response to O_2. Both proteins act as Trx-linked peroxidases capable of reducing NAD(P)H-dependent H_2O_2 and alkyl hydroperoxides in vitro (3, 8, 30). Two sets of thioredoxin-thioredoxin reductase genes are found in the *C. acetobutylicum* genome, one of which (*trxA1-trxB1-gpx3*) is up-regulated within 10 min after O_2 stress, while the other (*trxA2-trxB2*) is expressed constitutively. The actual electron transfer components to BCP and TSA remain to be identified; these two sets of thioredoxin-thioredoxin reductases are the possible electron donor proteins for these two peroxiredoxins. Furthermore, previously identified O_2-responsive rubrerythrins (NCBI accession no. NP_350180 and NP_350181) (35, 48) might be involved in NAD(P)H-dependent O_2, H_2O_2, or alkyl hydroperoxide reductase activities because the N-terminal parts of these rubrerythrins show high degrees of identity with rubredoxin, which is a small protein predicted to be involved in some electron transfer systems for oxygen and ROS metabolism (10, 11, 14, 22, 66). Proteins encoded by the O_2-responsive *nrror* gene cluster (Fig. 2A) are also considered to involve in NAD(P)H-dependent O_2 or ROS reductase activities.

These results suggest that not only oxygen metabolism but also active oxygen and lipid peroxide scavenging enzymes are important for the microoxic growth of *C. acetobutylicum*. We are now investigating the individual functions of the proteins encoded by O_2-responsive genes, the mechanisms of oxygen-responsive promoters, and the distribution of oxygen response systems among clostridia.

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