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Redox-Responsive Repressor Rex Modulates Alcohol Production and Oxidative Stress Tolerance in *Clostridium acetobutylicum*

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Rex, a transcriptional repressor that modulates its DNA-binding activity in response to NADH/NAD^+ ratio, has recently been found to play a role in the solventogenic shift of *Clostridium acetobutylicum*. Here, we combined a comparative genomic reconstruction of Rex regulons in 11 diverse clostridial species with detailed experimental characterization of Rex-mediated regulation in *C. acetobutylicum*. The reconstructed Rex regulons in clostridia included the genes involved in fermentation, hydrogen production, the tricarboxylic acid cycle, NAD biosynthesis, nitrate and sulfite reduction, and CO_2/CO fixation. The predicted Rex-binding sites in the genomes of *Clostridium* spp. were verified by *in vitro* binding assays with purified Rex protein. Novel members of the *C. acetobutylicum* Rex regulon were identified and experimentally validated by comparing the transcript levels between the wild-type and *rex*-inactivated mutant strains. Furthermore, the effects of exposure to methyl viologen or H_2O_2 on intracellular NADH and NAD^+ concentrations, expression of Rex regulon genes, and physiology of the wild type and *rex*-inactivated mutant were comparatively analyzed. Our results indicate that Rex responds to NADH/NAD^+ ratio *in vivo* to regulate gene expression and modulates fermentation product formation and oxidative stress tolerance in *C. acetobutylicum*. It is suggested that Rex plays an important role in maintaining NADH/NAD^+ homeostasis in clostridia.

Organisms of the genus *Clostridium* are Gram-positive obligate anaerobes important in human health and physiology, the carbon cycle, and biotechnological applications (1). As anaerobes, clostridia maintain the cellular redox balance mainly through the reactions of central metabolism. The reducing equivalents are generated through the glycolytic pathway and reoxidized through alcohol synthesis, hydrogen production, and other NADH-consuming reactions (Fig. 1) (2). To sustain growth and metabolism, the metabolic network must be operated to maintain the redox balance in the cell.

Among *Clostridium* species, *C. acetobutylicum* is one of the best-studied species and has been used to develop an industrial acetone, butanol, and ethanol (ABE) fermentation process (3, 4). The redox balance in *C. acetobutylicum* has been manipulated by using several approaches to push the metabolism toward butanol synthesis. These approaches include the addition of artificial electron carriers such as methyl viologen (MV) or neutral red, increasing the hydrogen partial pressure or gassing with carbon monoxide, and the utilization of reduced substrates like glycerol (2, 5). All these approaches are based on reducing hydrogen formation to provide a surplus of electron, i.e., NAD(P)H, for butanol synthesis. A recent transcriptomic study has gained first insights on the molecular level into the effect of MV addition to cultures of *C. acetobutylicum* (6). Although some interesting results have been obtained from these studies, the molecular regulatory mechanisms remain to be elucidated.

The strictly anaerobic clostridia have evolved mechanisms to survive limited exposure to air (7). To cope with the oxidative stress, clostridia express genes encoding the components of the detoxification system, which essentially include flavodiiron proteins, desulfoferrodoxin, and rubrerythrins (8). Clostridia use their reducing equivalents to reduce the toxic reactive oxygen species (ROS) and molecular O_2, thereby protecting crucial oxygen-sensitive metabolic enzymes (9). To generate the required reducing equivalents, the cellular redox balance needs to be shifted accordingly.

Recently, the redox-sensing transcriptional repressor Rex has been found to play a role in the solventogenic shift of *C. acetobutylicum* (10). Rex was first discovered in *Streptomyces coelicolor* and is widely distributed among Gram-positive bacteria. In *S. coelicolor* and *Bacillus subtilis*, Rex controls expression of cytochrome *bd* terminal oxidase and NADH dehydrogenase of the respiratory chain (11, 12). The Rex ortholog in *Staphylococcus aureus* regulates genes involved in anaerobic respiration and fermentation, such as lactate, formate, and ethanol formation and nitrate respiration (13). In *Streptococcus mutans* and *Enterococcus faecalis*, Rex has been shown to be involved in regulation of oxidative stress responses and to influence H_2O_2 accumulation, respectively (14, 15). The DNA-binding activity of Rex proteins is modulated by the ratio of NADH to NAD^+ concentrations (11, 16). The crystal structures of Rex proteins from *Thermus aquaticus* and *B. subtilis* in complex with NADH, NAD^+, and/or DNA operator have been determined (17, 18). Rex is composed of two domains, an N-terminal winged-helix DNA-binding domain and a C-terminal Rossmann-like domain involved in NADH binding and subunit dimerization.

Although the relative levels of NADH and NAD^+ have been shown to influence the DNA-binding activity of Rex based on *in*

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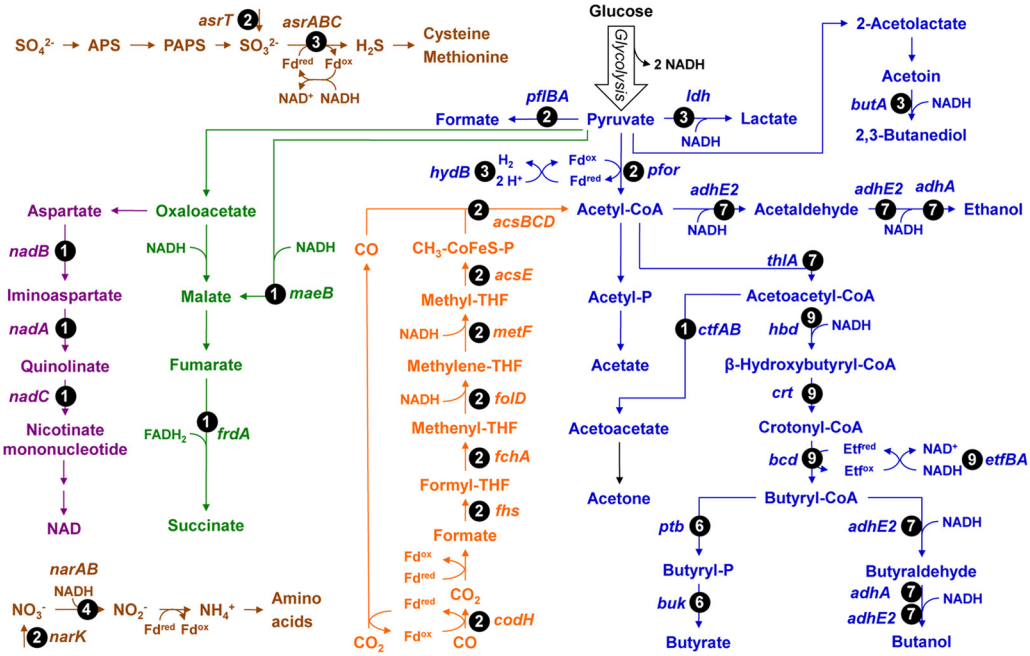

FIG 1 Metabolic context of the reconstructed Rex regulons in clostridia. The metabolic pathways are color coded as follows: fermentation, blue; Wood-Ljungdahl pathway, orange; (incomplete) TCA cycle, green; NAD biosynthesis, purple; nitrate and sulfate reduction, brown. Numbers in circles indicate the numbers of genomes where the target gene is preceded by a candidate Rex-binding site.

vitro binding assays, it remains unclear whether Rex monitors the NADH/NAD^+ ratio *in vivo* to control gene expression. Several genes associated with fermentation pathways have been identified as Rex targets in *C. acetobutylicum* (10). However, whether Rex also regulates transcription of other genes is not known. The role of Rex-dependent regulation in *C. acetobutylicum* in response to an altered cellular redox balance such as increased NAD(P)H availability or oxidative stress has not been studied. Moreover, although Rex seems to be widely distributed in clostridia, little is known about its targets and function in the species other than *C. acetobutylicum*.

In this study, we used a comparative genomic approach to reconstruct Rex regulons in 11 diverse clostridial species. These *Clostridium* species included the solvent-producing *C. acetobutylicum* and *C. beijerinckii*; the organic acid-producing *C. butyricum* and *C. kluyveri*; the acetogens that grow on CO_2/CO/H_2, including *C. carboxidivorans* and *C. ljungdahlii*; and the cellulolytic *C. cellulovorans*. The important human pathogens *Clostridium botulinum*, *C. perfringens*, and *C. tetani*, as well as *C. novyi*, having potential therapeutic uses in cancers, were also included. The reconstructed clostridial Rex regulons contain the genes associated with important metabolic processes, including fermentation, hydrogen production, NAD biosynthesis, nitrate and sulfite reduction, and CO_2/CO fixation. Comparative analysis of reconstructed Rex regulons revealed considerable variations in the regulon content between the analyzed clostridia. The predicted Rex-binding sites in the genomes of *Clostridium* spp. were verified by *in vitro* binding assays. Novel members of the Rex regulon in *C. acetobutylicum* were identified and experimentally validated. Furthermore, the effects of exposure to MV or H_2O_2 on intracellular NADH and NAD^+ concentrations, expression of Rex regulon genes, and physiology were compared between the wild-type and *rex*-inactivated mutant strains. Our results indicate that Rex monitors NADH/NAD^+ ratio *in vivo* to regulate gene expression and modulates fermentation product formation and oxidative stress response in *C. acetobutylicum*.

MATERIALS AND METHODS

Bioinformatics tools and resources. Genome sequences of clostridia analyzed in this study were obtained from GenBank (http://www.ncbi.nlm.nih.gov/GenBank/). Identification of orthologs was performed using the BLAST tool provided by NCBI (19). Orthologs of the Rex protein from *C. acetobutylicum* ATCC 824 were identified with a 50% protein sequence identity threshold. The ClustalX (version 2.1) program was used for protein sequence alignments (20). Reconstruction of Rex regulons was performed using an established comparative genomics method (21) implemented in the RegPredict webserver (http://regpredict.lbl.gov) (22) and the Genome Explorer software (23). The previously identified Rex recognition DNA motif in *Clostridiaceae* (24) was used to scan the *Clostridium* genomes and identify candidate Rex-binding sites. Scores of candidate sites were calculated as the sum of positional nucleotide weights. The score threshold was defined as the lowest score observed in the training set. Genes with candidate upstream binding sites that have high scores and/or are conserved in two or more genomes were included in the Rex regulon. Candidate sites associated with new regulon members were added to the training set, and the respective position weight matrices describing the clostridial Rex-binding DNA motif were rebuilt to improve search accuracy. Functional annotations of the predicted regulon members were based on the SEED database (http://theseed.uchicago.edu/FIG/index.cgi) (25).

Bacterial strains and growth conditions. *C. acetobutylicum* strain ATCC 824, its mutant with the *rex* gene inactivation (*rex*::intron), and the *rex*-complemented strain (*rex*::intron pSY9-*rex*) were used in this study. *C. acetobutylicum* strains were precultured anaerobically on clostridial growth medium (CGM) (26) to exponential growth phase. The cultures were started with the same optical density at 600 nm (OD_{600}; ~0.02) and

performed at 37°C in triplicate in 60 ml of P2 minimal medium (27), which contains (per liter) 0.5 g of K_2HPO_4, 0.5 g of KH_2PO_4, 2.2 g of CH_3COONH_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $MnSO_4 \cdot H_2O$, 0.01 g of NaCl, 0.01 g of $FeSO_4 \cdot 7H_2O$, 1 mg of *p*-aminobenzoic acid, 1 mg of vitamin B_1, 0.01 mg of biotin, and 60 g of glucose. For methyl viologen-exposed cultures, MV was added to a final concentration of 1 mM when cells were grown in P2 minimal medium to an OD_{600} of about 0.15. For hydrogen peroxide challenge experiments, cells were grown in P2 minimal medium to an OD_{600} of about 2.0. Then, cells were exposed to 50, 100, or 200 μM H_2O_2 or the equal volume of H_2O. Because the sensitivity of *C. acetobutylicum* toward H_2O_2 was largely dependent on Fenton chemistry (28), 1 mM iron chelator 2,2′-dipyridyl (Sigma-Aldrich) was added to attenuate peroxide-dependent killing of cells. After incubation at 37°C for 30 min, the number of surviving cells was determined as described previously (29). Briefly, aliquots of appropriate dilutions were plated on CGM and incubated anaerobically for 36 h at 37°C. The CFU for each sample were determined and normalized to the number obtained for the non-stressed wild type (100%).

Mutant construction. Gene disruption in *C. acetobutylicum* ATCC 824 was performed by using group II intron-based targetron technology as described previously (30). Briefly, a 350-bp fragment for retargeting an intron to insert within the *rex* gene (CAC2713) was generated by one-step assembly PCR using the primers shown in Table S1 in the supplemental material according to the protocol of the TargeTron gene knockout system (Sigma). The PCR product was digested and ligated to targetron vector pWJ1 (31), yielding the plasmid pWJ1-*rex*. The plasmid was methylated *in vivo* in *Escherichia coli* ER2275(pAN1) (32) and electroporated into *C. acetobutylicum* ATCC 824. The transformants were selected on a CGM plate supplemented with erythromycin. The resulting mutant with an intron insertion in the *rex* gene was confirmed by PCR.

For genetic complementation experiments, the *rex* gene from *C. acetobutylicum* was cloned into the pSY9 vector (33) under the control of the constitutive P_{ptb} promoter (34). PCR was carried out using the *C. acetobutylicum* ATCC 824 genomic DNA and the primers shown in Table S1 in the supplemental material. The obtained plasmid pSY9-*rex* was electroporated into the *rex*-inactivated mutant, generating the *rex*-complemented strain.

RNA isolation and real-time PCR analysis. Total RNA was isolated from *C. acetobutylicum* ATCC 824 grown in the P2 minimal medium with or without addition of MV or H_2O_2. Cells were harvested at mid-exponential growth phase (OD_{600} of about 2.0), frozen immediately in liquid nitrogen, and ground into powder. RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. Contaminant DNA was removed by DNase I (TaKaRa) digestion. RNA (1 μg) was transcribed into cDNA with random primers using the ReverTra-Plus kit from Toyobo. The product was quantified via real-time PCR using the CFX96 thermal cycler (Bio-Rad). The reaction mixture (20 μl) contained Power SYBR green PCR master mix (Bio-Rad) and 0.4 μm gene-specific primers (as shown in Table S1 in the supplemental material). The PCR parameters were 1 cycle of 95°C for 2 min, followed by 40 cycles of 95°C for 20 s, 60°C for 20 s, and 72°C for 15 s. The accuracy of the PCR product was checked by melting curve analysis. The expression level of each gene was normalized with the value for the CAC2679 gene encoding a pullulanase, which was used as a reference gene with constitutive expression (35). Data were presented as the average of six measurements from two biological replicates, with the corresponding standard deviation.

Protein overexpression and purification. The *rex* (CAC2713) gene was PCR amplified from *C. acetobutylicum* ATCC 824 genomic DNA using the primers shown in Table S1 in the supplemental material. The PCR fragment was ligated into the expression vector pET28a cleaved by BamHI and SalI. The resulting plasmid pET28a-*rex* was used to produce Rex protein with an N-terminal hexahistidine tag. The plasmid pET28a-*rex*-Q51K coding for a Rex mutant where the glutamine residue Gln51 was replaced by a lysine residue was constructed with two steps of PCR using pET28a-*rex* as the template and the mutagenic primers and flanking primers (see Table S1). All recombinant plasmids were sequenced to exclude unwanted mutations in the *rex* gene. For overproduction of Rex protein and its mutated derivative, *E. coli* BL21(DE3)pLysS (Novagen) was transformed with expression plasmid pET28a-*rex* or pET28a-*rex*-Q51K and cultivated in LB medium at 37°C to an OD_{600} of 0.8. Protein expression was induced by the addition of 0.2 mM isopropyl-β-D-thiogalactopyranoside, and the culture was incubated for another 12 h at 16°C. After the cells were harvested, purification of Rex by nickel-nitrilotriacetic acid affinity chromatography was performed as described previously (36). The purified protein was run on a 12% sodium dodecyl sulfate-polyacrylamide gel to monitor its size and purity.

Electrophoretic mobility shift assay (EMSA). The 180-bp DNA fragments in the promoter region of individual genes were PCR amplified using the primers shown in Table S1 in the supplemental material. Both forward and reverse primers were Cy5 fluorescence labeled at the 5′ end (Sangong Corp., Shanghai, China), and the PCR products were purified with a PCR purification kit (Axygen). Purified Rex protein or its mutated derivative was incubated with the fluorescence-labeled DNA fragment (1 nM) in 20 μl of binding buffer containing 20 mM Tris (pH 7.5), 0.25 mM dithiothreitol (DTT), 10 mM $MgCl_2$, 5% glycerol, 0.8 μg bovine serum albumin (BSA), and 1 μg salmon sperm DNA (nonspecific competitor). Promoter fragments lacking a putative Rex-binding site were used as negative controls. As potential effectors of Rex-DNA binding, NADH and/or NAD^+ was added as indicated. After incubation at 30°C for 20 min, the reaction mixture was electrophoresed at 4°C on a 6% native polyacrylamide gel in 0.5× Tris-borate-EDTA for 1.5 h at 100 V. Fluorescence-labeled DNA on the gel was then detected with the Starion FLA-9000 scanner (FujiFilm, Japan). For the determination of apparent dissociation constants (K_d), the bands were quantified using Quantity One software, and the percentage of shifted DNA was calculated. These values were plotted against the Rex concentration, and K_d values were obtained using the GraphPad Prism software. All determinations were performed at least in triplicate.

Metabolite analysis. For analysis of extracellular metabolites, culture samples were centrifuged for 10 min at 4°C and 15,000 × *g* to remove the cells. Acetone, ethanol, and butanol were detected by a gas chromatograph (GC) (Agilent model 7890A) equipped with a capillary column (Alltech EC-Wax; 30 m by 0.32 mm) and a flame ionization detector (Agilent).

The intracellular NADH and NAD^+ were extracted and assayed by using the fluorescent NAD^+/NADH detection kit (Cell Technology Inc., CA), which utilizes a nonfluorescent detection reagent that is reduced in the presence of NADH to produce its fluorescent analog. Briefly, cells were harvested at mid-exponential growth phase (OD_{600} of about 2.0) by centrifuging 2 ml of culture broth at 9,000 × *g* and 4°C for 10 min. Intracellular NADH and NAD^+ were extracted using respective extraction buffers by following the manufacturer's instructions. NADH reacted with nonfluorescent detection reagent to form NAD^+ and the fluorescent analog. The concentration of the formed fluorescent analog was then determined at 550-nm excitation and 595-nm emission wavelengths by using a spectrofluorometer (Varioskan Flash; Thermo Scientific Co.). NAD^+ is further converted to NADH via an enzyme-coupled reaction. The enzyme reaction specifically reacts with NAD^+/NADH and not with $NADP^+$/NADPH. A series of NADH and NAD^+ standards were used to obtain a calibration curve for determining the concentrations of these compounds in the cell extracts. The intracellular NADH and NAD^+ concentrations were then calculated by normalization to cell volume. A predetermined correlation factor of 0.26 g (dry weight) of cells per OD_{600} and a previously reported intracellular aqueous volume of 1.67 μl per mg (dry weight) of cells (37) were used for calculation. Data were presented as the average of nine measurements from three biological replicates, with the corresponding standard deviation.

RESULTS

Comparative genomic reconstruction of Rex regulons in *Clostridium* spp. To reconstruct the Rex regulons in *Clostridium* species, we applied the integrative comparative genomics approach that combines identification of candidate transcription factor-binding sites with cross-genomic comparison of regulons and with the functional context analysis of candidate target genes. The analyzed clostridia include *C. acetobutylicum*, *C. beijerinckii*, *C. botulinum*, *C. butyricum*, *C. kluyveri*, *C. novyi*, *C. perfringens*, *C. tetani*, *C. cellulovorans*, *C. carboxidivorans*, and *C. ljungdahlii*. These 11 species with complete genome sequences belong to *Clostridium* cluster I (38), whereas they exhibit markedly different phenotypes (for example, they include saccharolytic and proteolytic species as well as solventogenic and acetogenic species). Rex proteins in these clostridia share close sequence homology (>68% identity), and particularly the sequences of the N-terminal DNA-binding domain are highly conserved (see Fig. S1 in the supplemental material). The previously identified Rex-binding DNA motif in *Clostridiaceae* (24), which has consensus TTGTTAANN NNTTAACAA, was used to search for Rex-binding sites in the genomes of *Clostridium* species. Finally, we performed a cross-species comparison of the predicted sets of potentially coregulated genes to define the Rex regulon for each species. The candidate members and metabolic context of the Rex regulons in the 11 *Clostridium* species are shown in Table 1 and Fig. 1, respectively. Detailed information about the predicted DNA-binding sites, candidate Rex target genes, and their known or predicted transcriptional start sites is provided in Table S2 in the supplemental material.

The reconstructed Rex regulons control the fermentation in all analyzed clostridia (Fig. 1 and Table 1). Most of the predicted Rex targets encode enzymes that consume NADH or other reducing equivalents (e.g., reduced ferredoxin). However, the size and the specific content of reconstructed Rex regulons are highly variable between different clostridial species. For instance, the Rex regulon in *C. beijerinckii* constitutes 11 operons, whereas in *C. kluyveri* Rex is predicted to control only one operon. Based on distribution of predicted Rex-regulated genes in *Clostridium* species, we classified them into the conserved and variable parts of Rex regulons. The conserved part of the Rex regulons includes 5 operons that are potentially regulated by Rex in at least 6 species. They are the *adhA* gene encoding alcohol dehydrogenase, the *adhE2* gene encoding bifunctional alcohol/acetaldehyde dehydrogenase, the *thlA* gene and *crt-bcd-etfBA-hbd* operon responsible for the conversion of acetyl coenzyme A (acetyl-CoA) to butyryl-CoA, and the *ptb-buk* operon for butyrate synthesis. On the other hand, 15 target operons form a group of species-specific regulon members that are preceded by candidate Rex-binding sites in at most 3 genomes analyzed. This group includes the genes involved in fermentation (*ldh*, *pflBA*, *ctfAB*, *butA*, and Cbei_4318), hydrogen production (*hydB*), tricarboxylic acid (TCA) cycle (*frd* and *maeB*), and nitrate and sulfite reduction (*narK*, *asrABC*, and *asrT*). In addition, the NAD biosynthetic genes *nadABC* were identified as candidate members of the Rex regulon in *C. acetobutylicum*. Regulation of the Wood-Ljungdahl pathway (*codH-cooC-fhs-fchA-folD-metF-lpdA-cooC-acsDCEB*) by Rex, which is used to fix CO_2 or CO, was predicted for *C. carboxidivorans* and *C. ljungdahlii*.

In summary, the comparative genomics analysis allowed us to reconstruct the Rex regulons in 11 diverse clostridial species. Among these species, *C. acetobutylicum* has one of the largest sets of Rex targets, including 17 genes organized in 7 operons that contain not only the known targets (*ldh*, *adhE2*, *thlA*, and *crt-bcd-etfBA-hbd*) but also the newly identified members (*ptb-buk*, *nadABC*, and *asrTABC*). These Rex targets are involved in fermentation, NAD biosynthesis, and sulfite reduction. We then performed experimental characterization of the clostridial Rex-binding motif and the Rex-mediated regulation in *C. acetobutylicum* as described below.

Rex binds to the promoter regions of predicted target genes *in vitro*. To validate the predicted clostridial Rex regulons, electrophoretic mobility shift assays (EMSAs) were performed using the recombinant Rex from *C. acetobutylicum*, which was overexpressed in *E. coli* with the N-terminal His_6 tag and purified with a nickel-chelating affinity column. For all predicted Rex target operons in *C. acetobutylicum*, DNA fragments (180 bp) in the promoter regions containing candidate Rex-binding sites were tested in EMSAs (Fig. 2A). A shifted band was observed upon incubation of Rex protein with each promoter fragment, and its intensity was enhanced in the presence of increasing amounts of Rex protein. As a negative control, the promoter fragment of *pflBA* operon in *C. acetobutylicum*, which lacks a predicted Rex-binding site, was used, and no binding was observed even at 3,000 nM Rex protein (Fig. 2B). The formation of Rex-DNA complex was suppressed in the presence of 400-fold excess unlabeled DNA fragments but not in the presence of nonspecific competitor, salmon sperm DNA (data not shown). These results confirm that Rex binds specifically to the promoter regions of the predicted Rex target operons in *C. acetobutylicum*.

The apparent dissociation constant (K_d) values of Rex protein interacting with the tested *C. acetobutylicum* DNA fragments were determined, and they varied in a wide range from 23 nM to 393 nM (Fig. 2). According to the K_d values, the tested DNA fragments can be divided into two groups. For the first group including the promoter fragments of *adhE2*, *ldh* genes, and the *crt-bcd-etfBA-hbd* operon, Rex protein exhibited a high affinity and the K_d values were in the range of 23 to 37 nM. The second group includes the fragments from the promoter regions of the *thlA* gene and the *asrTABC*, *ptb-buk*, and *nadABC* operons. The K_d values for this group were in the range of 177 to 393 nM, indicating a lower affinity of Rex to these target fragments.

EMSAs were also performed to assess the predicted Rex-binding sites in other analyzed clostridia. For each predicted Rex target operon, the upstream candidate Rex-binding site in one or two genomes was tested (Table 1). Thus, 16 DNA fragments were amplified from the promoter regions of *C. beijerinckii pflBA*, *adhA*, *adhA2*, *butA*, *hydB*, and *fld*-Cbei_4318; *C. botulinum maeB*; *C. novyi ctfAB*; *C. perfringens noxE*; *C. tetani frdA*; *C. carboxidivorans narAB*, *narK*, *codH-cooC-fhs-fchA-folD-metF-lpdA-cooC-acsDCEB*, and *grdIH*; and *C. ljungdahlii codH* and *bcd2*, respectively. These DNA fragments were tested for binding of *C. acetobutylicum* Rex protein that is well conserved in the analyzed clostridia. A shift in the presence of purified Rex was observed for all the 16 fragments (Fig. 3A and B). For the *C. carboxidivorans narK* fragment, two shifted Rex-DNA complexes were detected, supporting our prediction that two DNA-binding sites are present (see Table S2 in the supplemental material). Most of the promoter fragments were completely shifted with 1,000 nM Rex (Fig. 3A and B). In contrast, the promoter fragments of the *C. beijerinckii ldh* gene and the *C. carboxidivorans crt-hbd-thlA-bcd-etfBA* operon, which do not

TABLE 1 Rex regulons in 11 species of clostridia[a]

Operon and metabolism	Presence of gene in *Clostridium* species: *C. acetobutylicum* ATCC 824	*C. beijerinckii* NCIMB 8052	*C. botulinum* ATCC 3502	*C. butyricum* 5521	*C. kluyveri* DSM 555	*C. novyi* NT	*C. perfringens* ATCC 13124	*C. tetani* E88	*C. cellulovorans* 743B	*C. carboxidivorans* P7	*C. ljungdahlii* DSM 13528	Functional role
Fermentation												
ldh	**+***	−	+	−	−	−	+	−	0	0	0	L-Lactate dehydrogenase
pflBA	−	**+***	−	−	−	−	−	0	+	0	0	Pyruvate formate-lyase
adhA	0	**+***	+	+	−	+	−	+	−	+	+	Alcohol dehydrogenase [Fe]
adhE2	**+***	−	+	+	−	0	+	+	+	+	−	Alcohol/acetaldehyde dehydrogenase
thlA	**+***	+	+	+	−	+	+	+	−	−	0	Acetyl-CoA acetyltransferase
crt-bcd-etfBA-hbd	**+***	+	+	+	+	+	+	+	+	−	0	Butyryl-CoA synthesis enzymes
ctfAB	−	−	−	0	0	**+***	0	0	0	0	0	CoA-transferase
ptb-buk	**+***	+	−	+	0	0	+	+	−	+	0	Phosphotransbutyrylase, butyrate kinase
butA	0	**+***	+	+	0	−	0	0	0	−	−	2,3-Butanediol dehydrogenase
hydB	0	**+***	−	−	0	+	−	+	0	−	−	Fe-hydrogenase
fld-Cbei_4318	−	**+***	−	+	−	−	−	−	0	0	−	Flavodoxin, pyruvate flavodoxin/ferredoxin oxidoreductase
TCA cycle												
frdA	0	0	0	0	0	0	0	**+***	0	0	0	Fumarate reductase flavoprotein subunit
maeB	0	−	**+***	+	0	0	0	0	0	−	−	Malic enzyme
NAD biosynthesis												
nadABC	**+***	−	−	−	−	−	−	0	−	−	−	NAD biosynthesis enzymes
Nitrate and sulfite reduction												
narAB	0	0	0	+	0	0	+	0	0	**+***	+	Nitrate reductase
narK	0	0	0	0	0	0	−	0	0	**+***	+	Nitrate/nitrite transporter
asrABC	**+***	−	−	−	0	−	−	−	−	+	+	Sulfite reductase
asrT	**+***	−	−	0	0	0	−	−	−	+	0	Predicted sulfite/sulfate transporter
Wood-Ljungdahl pathway												
codH-cooC-fhs-fchA-folD-metF-lpdA-cooC-acsDCEB	0	0	0	0	0	0	0	0	0	**+***	**+***	Wood-Ljungdahl pathway enzymes
Other												
grdIH	0	0	0	0	0	0	0	0	0	**+***	+	Betaine/glycine reductase
noxE	0	−	+	−	0	+	**+***	+	0	0	0	NADH oxidase
bcd2-fldA-bcd-etfBA	0	0	0	0	0	0	0	0	0	0	**+***	Acyl-CoA dehydrogenase, acyl-CoA transferase, flavoprotein

[a] The genes preceded by a conserved Rex-binding site are indicated by +, and the predicted Rex-binding sites verified by targeted experiments are marked by bold type and an asterisk. Genes without a candidate Rex-binding site are indicated by −. The absence of an orthologous gene(s) in the analyzed genomes is indicated by 0.

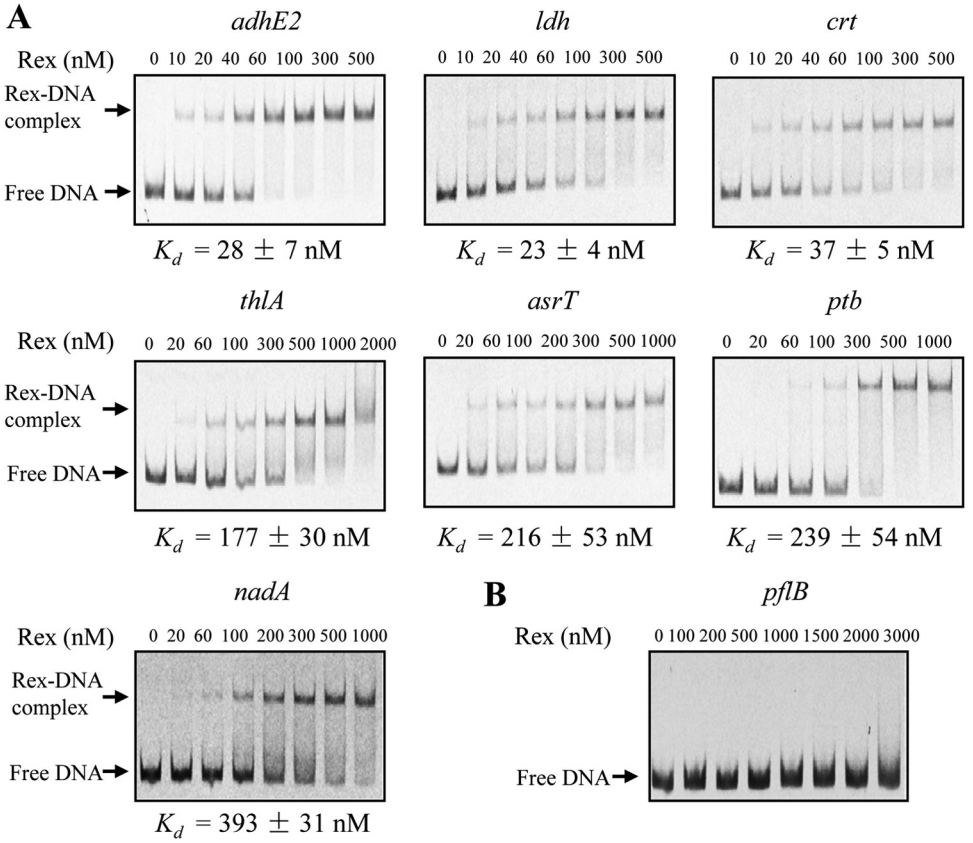

FIG 2 EMSAs with purified Rex protein and DNA fragments from the promoter regions of predicted target genes in *C. acetobutylicum*. (A) DNA fragments (1 nM) from the promoter regions of *C. acetobutylicum adhE2*, *ldh*, *crt*, *thlA*, *asrT*, *ptb*, and *nadA* genes were fluorescence labeled and incubated with the indicated concentrations of Rex protein for 20 min at 30°C. Then, the protein-DNA complexes were resolved by electrophoresis on native 6% polyacrylamide gels. Quantification of the bands allowed the determination of the apparent K_d values (see Materials and Methods). The values shown represent the average and standard deviation of at least three independent assays. (B) As a negative control, the promoter region of the *C. acetobutylicum pflBA* operon, which lacks a putative Rex-binding site, was used.

contain predicted Rex-binding sites, were not shifted even with 3,000 nM Rex protein (Fig. 3C).

Characterization of the Rex-binding motif in clostridia. The identified clostridial Rex-binding motif has the consensus TTGT TAANNNNTTAACAA, which deviates in two positions (i.e., positions 5 and 14) from the common consensus TTGTGAANNNN TTCACAA of the Rex-binding motifs in most Gram-positive bacteria such as *B. subtilis* and *S. coelicolor* (24). Among the confirmed Rex-binding sites in clostridia, the thymine at position 5 and adenine at position 14 are highly conserved (Fig. 4A). For characterization of the Rex-binding motif in clostridia, mutational analysis was performed on the promoter fragment of the *C. acetobutylicum crt-bcd-etfBA-hbd* operon. This operon is a conserved Rex regulon member in clostridia, and its promoter fragment showed a substantial shift in the presence of 40 nM Rex (Fig. 2A). We changed the thymine at position 5 to guanine or/and the adenine at position 14 to cytosine to match the common Rex consensus sequence in Gram-positive bacteria (Fig. 4A). The mutated fragments were amplified by PCR and tested in EMSAs for binding of *C. acetobutylicum* Rex (Fig. 4B). Substitution of the thymine 5 or adenine 14 in the Rex-binding site increased the apparent K_d value of Rex about 10-fold and 2-fold, respectively. The reduced binding affinity of Rex to the mutated Rex-binding sites indicates that the thymine 5 and adenine 14 in the operator are important for Rex binding in clostridia.

In the Rex proteins, Lys47 is a relatively conserved residue in the DNA recognition helix, and it forms a hydrogen bond with guanine 5 of the DNA operator according to a structural study of *T. aquaticus* Rex (17). However, the Lys47 residue was mutated to glutamine (Gln51) in the Rex proteins from *Clostridium* spp. (see Fig. S1 in the supplemental material). To assess if this residue substitution influences the Rex-DNA contacts, the Gln51 residue of *C. acetobutylicum* Rex was changed to a lysine residue by site-directed mutagenesis. The resulting Rex variant was overproduced in *E. coli*, purified, and used for EMSAs. As shown in Fig. 4C, the mutated protein Rex-Q51K exhibited a 12-fold-increased apparent K_d value for the promoter fragment of the *crt-bcd-etfBA-hbd* operon. Nevertheless, when the thymine 5 or adenine 14 in the binding sequence was changed to guanine and cytosine, respectively, the binding affinity of the mutated protein Rex-Q51K was significantly increased. Therefore, these results indicate a correlation between a key amino acid residue in the DNA-binding domain of Rex proteins and two nucleotides at symmetrical positions of the palindromic Rex-binding motifs. For clostridial Rex proteins, the Gln51 residue in the recognition helix might position within the major groove of DNA and contact the thymine 5 and

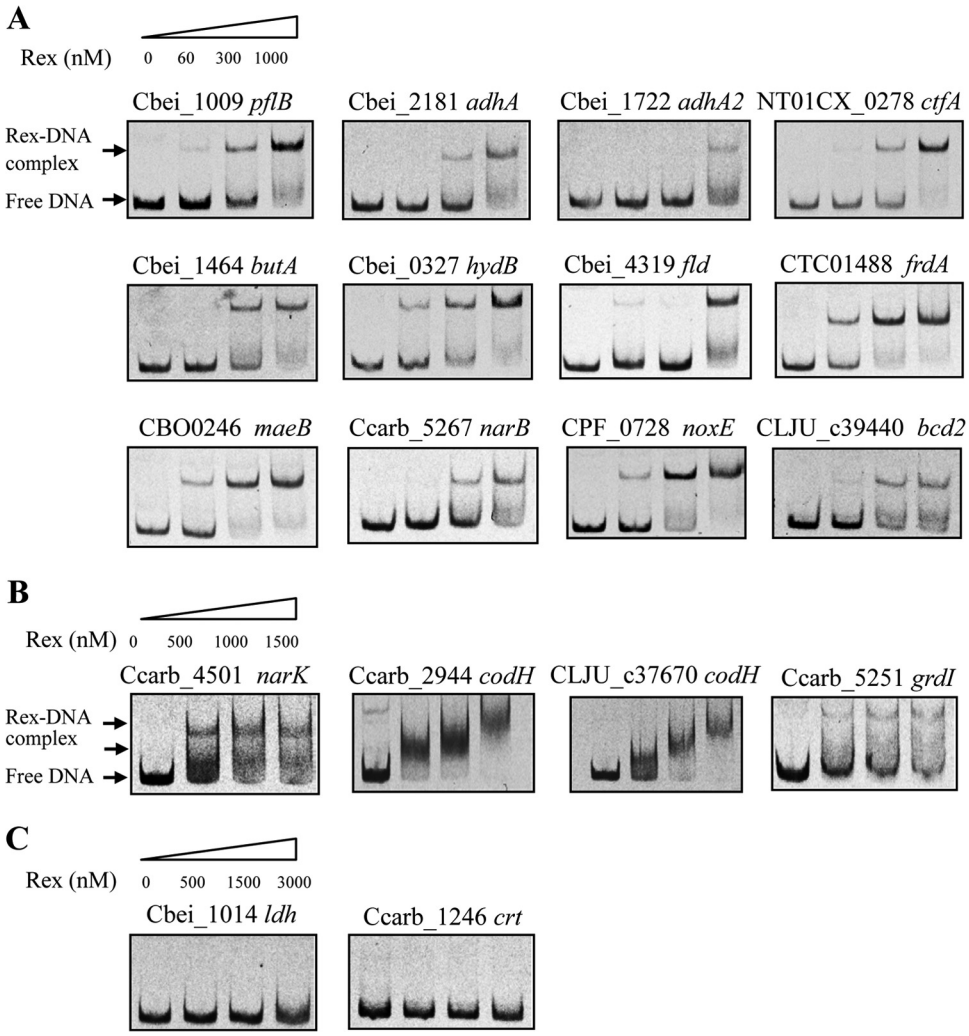

FIG 3 EMSAs with purified Rex protein and the promoter regions of predicted target genes in *Clostridium* species other than *C. acetobutylicum*. (A) EMSAs were performed in the absence (lanes 1) and in the presence of 60, 300, and 1,000 nM Rex protein (lanes 2 to 4, respectively). (B) EMSAs were performed in the absence (lanes 1) and in the presence of 500, 1,000, and 1,500 nM Rex protein (lanes 2 to 4, respectively). (C) The negative controls included the *ldh* promoter of *C. beijerinckii* and the *crt* promoter of *C. carboxidivorans*, which do not contain the predicted Rex-binding site.

adenine 14 of DNA operators (see Fig. S2 in the supplemental material).

Effect of NADH and NAD$^+$ on Rex-DNA interactions. To test if NADH and NAD$^+$ affect the interaction between the Rex from *C. acetobutylicum* and its cognate operators, EMSAs were performed using the promoter fragment of the *adhE2* gene. As shown in Fig. 5A, the presence of only 5 μM NADH drastically decreased the formation of Rex-DNA complex, whereas the addition of 1 mM NAD$^+$ results in a noticeable enhancement of Rex binding to the DNA fragment (Fig. 5A, lanes 3 and 4). This effect is specific for NADH and NAD$^+$, as it was not found for 10-fold-higher concentrations of NADPH and NADP$^+$ (Fig. 5A, lanes 7 and 8). Furthermore, interaction between the same DNA fragment and *C. acetobutylicum* Rex protein was assessed in the presence of physiological concentrations of NADH and NAD$^+$. The intracellular NADH and NAD$^+$ pool sizes in *C. acetobutylicum* are decreased from 0.3 mM and 1.6 mM, respectively, during the exponential growth phase, to 0.1 mM and 1.2 mM, respectively, during the subsequent solventogenic phase (39). Thus, various concentrations of NADH and NAD$^+$, which cover the physiological concentration ranges, were used in EMSAs. As shown in Fig. 5B, the DNA-binding activity of Rex was particularly susceptible to changes in the NADH concentration, but NAD$^+$ clearly influenced the inhibitory effect of NADH on Rex-DNA complex formation. These results strongly suggest that *C. acetobutylicum* Rex senses and responds to the intracellular ratio of NADH to NAD$^+$ to modulate its DNA-binding activities under physiological conditions.

Rex negatively regulates expression of its direct target genes *in vivo*. To validate the predicted regulation of Rex on gene expression *in vivo*, the *rex* gene in *C. acetobutylicum* was disrupted by insertion of an intron, resulting in the *rex*-inactivated mutant (confirmed by PCR as shown in Fig. S3 in the supplemental material). The transcript levels of the predicted Rex direct targets in the *rex*-inactivated mutant were compared with those in the wild type by using quantitative real-time-PCR (qRT-PCR). The two strains were cultivated in minimal medium with 60 g liter^{-1} of glucose as a carbon source, and no differences in cell growth were observed for them. For comparison of transcript levels, cells were

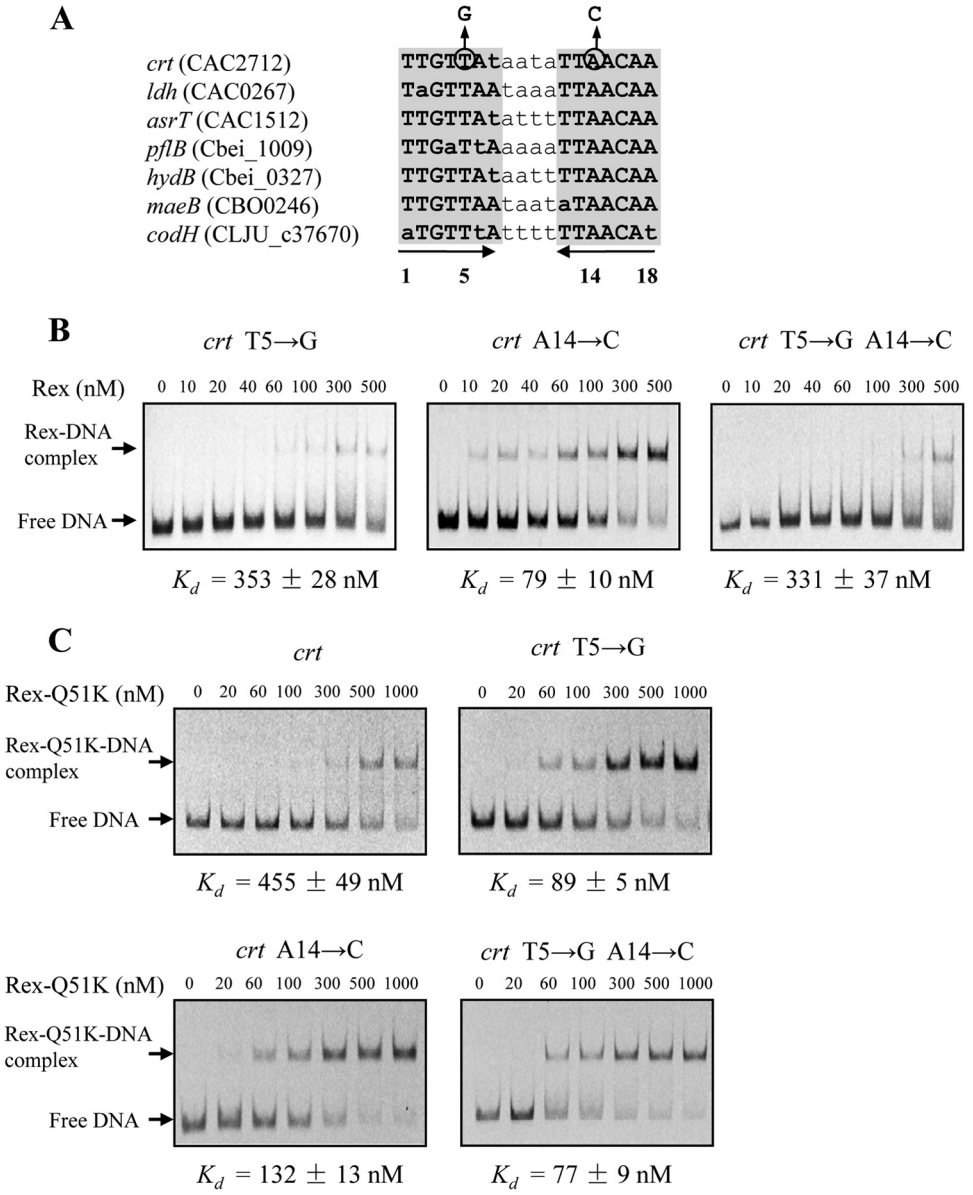

FIG 4 Characterization of the Rex-binding motif in clostridia. (A) Alignment of the Rex-binding sites in the promoter regions of the *C. acetobutylicum crt-bcd-etfBA-hbd* operon, *ldh* gene, and *asrTABC* operon; the *C. beijerinckii pflB* and *hydB* genes; the *C. botulinum maeB* gene; and the *C. ljungdahlii codH-cooC-fhs-fchA-folD-metF-lpdA-cooC-acsDCEB* operon. The palindromic sequences are shaded. The conserved nucleotides are shown in bold capitals. Bases substituted in the *crt* promoter for EMSAs are indicated, and the new base is shown above. (B) Mutational analysis of the Rex-binding site in the *crt* promoter of *C. acetobutylicum*. The mutations were introduced by PCR, and the corresponding DNA fragments were analyzed by EMSAs with purified Rex protein. The apparent K_d values were determined as described in Materials and Methods. (C) Effect of mutagenesis of Rex on Rex-DNA interactions. A Rex derivative (Rex-Q51K) obtained by site-directed mutagenesis was used in EMSAs to test for binding to the *crt* promoter and mutated fragments.

harvested in the exponential growth phase at an OD_{600} of 2.0 and a growth rate of 0.16 h^{-1} for both strains, and total RNA was isolated. Six qRT-PCR measurements from two independent cultures were performed. As shown in Table 2, the relative mRNA levels of all the 17 genes were elevated more than 1.5-fold in the *rex*-inactivated mutant compared with the wild-type strain. The most prominent effect of *rex* mutation was observed for the *adhE2* gene, which showed a ≥160-fold-increased mRNA level in the *rex*-inactivated mutant. Complementation of the *rex*-inactivated mutant by using a plasmid construct constitutively expressing *rex* reduced the *adhE2* gene expression (see Fig. S3). The genes with a strongly increased expression in the *rex*-inactivated mutant also include the *ldh* and *thlA* genes (Table 2). The *crt-bcd-etfBA-hbd*, *asrTABC*, and *nadABC* operons showed a 1.5- to 3-fold-elevated transcript level in the *rex*-inactivated mutant. Expression of the *ptb-buk* operon was also increased by *rex* mutation. Therefore, the qRT-PCR results confirm that Rex is a negative regulator of *ldh*, *adhE2*, *thlA*, *crt-bcd-etfBA-hbd*, *ptb-buk*, *nadABC*, and *asrTABC* operons involved in fermentation, NAD biosynthesis, and sulfite reduction in *C. acetobutylicum*.

Rex plays a role in maintaining NADH/NAD^+ homeostasis in *C. acetobutylicum*. To understand the role of Rex-dependent

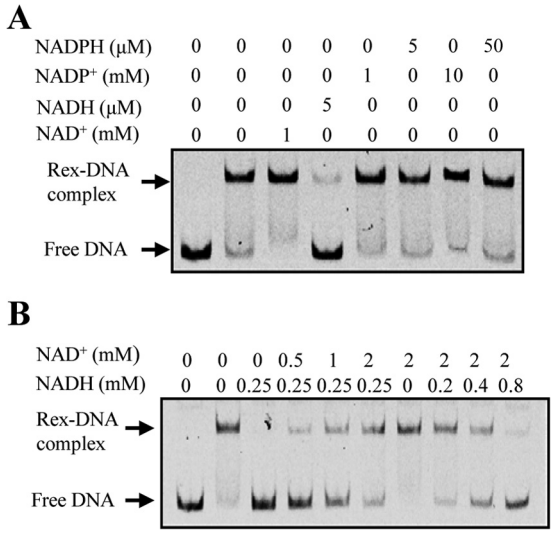

FIG 5 Effect of NADH and NAD^+ on the DNA-binding activity of Rex. (A) EMSAs were performed using *C. acetobutylicum adhE2* promoter fragment (1 nM), Rex protein (60 nM), and the indicated concentrations of pyridine nucleotides. No protein was added to the first lane. (B) EMSAs were performed as in panel A but with a range of physiological concentrations of NADH and NAD^+.

regulation in *C. acetobutylicum*, we investigated the effects of exposure of the wild-type and *rex*-inactivated mutant strains to methyl viologen (MV) or hydrogen peroxide (H_2O_2). First, the effects on intracellular NADH and NAD^+ concentrations were determined. The strains were cultivated in minimal medium without or with addition of 1 mM MV or 30 μM H_2O_2 and harvested in the exponential growth phase at an OD_{600} of about 2.0. Quantification of intracellular NADH and NAD^+ concentrations revealed an increase in the size of the total NAD pool in the *rex*-inactivated mutant compared to the wild type, which could be due to derepression of NAD biosynthetic genes in the mutant (Table 3). In accordance with previous findings (40), exposure of the wild type to MV caused a 2.5-fold increase in the NADH/NAD^+ concentration ratio (Table 3). Although the NADH/NAD^+ ratios were similar in the wild type and *rex*-inactivated mutant grown in cultures without MV addition, MV-exposed *rex*-inactivated mutant exhibited a 1.5-fold-increased NADH/NAD^+ ratio compared to MV-exposed wild type. In contrast, the intracellular NADH/NAD^+ ratio was decreased by 21% and 56% in the wild-type and *rex*-inactivated mutant, respectively, by H_2O_2 addition (Table 3). Therefore, MV addition resulted in a remarkable increase in intracellular NADH/NAD^+ ratio, whereas exposure to H_2O_2 significantly reduced the NADH/NAD^+ ratio. The *rex*-inactivated mutant showed larger fluctuations in the NADH/NAD^+ ratio than did the wild type when exposed to MV or H_2O_2, suggesting that Rex plays an important role in maintaining NADH/NAD^+ homeostasis in *C. acetobutylicum*.

TABLE 2 Comparison of mRNA levels in *C. acetobutylicum* wild type and *rex*-inactivated mutant using qRT-PCR

Gene name	Gene identifier	mRNA ratio[a] (*rex* mutant/WT[b])
adhE2	CAP0035	164.75 ± 6.45
ldh	CAC0267	13.75 ± 0.84
thlA	CAC2873	12.46 ± 2.34
crt	CAC2712	2.43 ± 0.10
bcd	CAC2711	2.81 ± 0.78
etfB	CAC2710	2.17 ± 0.20
etfA	CAC2709	1.55 ± 0.14
hbd	CAC2708	2.79 ± 0.37
ptb	CAC3076	6.07 ± 1.35
buk	CAC3075	1.97 ± 0.12
nadA	CAC1025	2.53 ± 0.38
nadB	CAC1024	1.79 ± 0.12
nadC	CAC1023	1.79 ± 0.26
asrT	CAC1512	2.04 ± 0.72
asrA	CAC1513	2.41 ± 0.74
asrB	CAC1514	2.67 ± 0.36
asrC	CAC1515	1.69 ± 0.48

[a] Data represent means ± standard deviations of values of mRNA ratios obtained from six measurements starting from two independent cultures. The strains were cultivated in P2 minimal medium, and total RNA was isolated in the exponential growth phase at an OD_{600} of about 2.0. The *P* value of the mRNA ratios for all the genes studied is smaller than 0.01.
[b] WT, wild type.

TABLE 3 Intracellular NADH and NAD^+ concentrations in *C. acetobutylicum* wild type and *rex*-inactivated mutant[a]

Strain	Addition to cultures	NADH concn (mM)	NAD^+ concn (mM)	NADH/NAD^+ ratio
Wild type		0.19 ± 0.01	0.83 ± 0.01	0.23 ± 0.02
Wild type	MV	0.27 ± 0.03	0.46 ± 0.01	0.59 ± 0.05
Wild type	H_2O_2	0.20 ± 0.02	1.11 ± 0.11	0.18 ± 0.02
rex mutant		0.26 ± 0.02	1.02 ± 0.18	0.25 ± 0.02
rex mutant	MV	0.34 ± 0.08	0.38 ± 0.07	0.92 ± 0.11
rex mutant	H_2O_2	0.16 ± 0.02	1.44 ± 0.12	0.11 ± 0.02

[a] Data represent means ± standard deviations of nine measurements from three biological replicates. The strains were grown in the P2 minimal medium without or with addition of 1 mM MV or 30 μM H_2O_2. The intracellular concentrations of NADH and NAD^+ were determined in the exponential growth phase at an OD_{600} of about 2.0.

Rex monitors NADH/NAD^+ ratio *in vivo* to regulate gene expression. The effect of exposure to MV or H_2O_2 on expression of genes in the Rex regulon was compared between *C. acetobutylicum* wild-type and *rex*-inactivated mutant strains. The transcript levels of the genes involved in fermentation were determined by using qRT-PCR, because the fermentation genes comprise the major direct targets of clostridial Rex. As shown in Fig. 6A, expression of Rex target genes *adhE2*, *thlA*, *crt*, *bcd*, and *hbd* in the wild type was significantly upregulated by MV addition. Most strikingly, the *adhE2* gene in the wild type showed an 80-fold-increased mRNA level in the presence of MV, which is consistent with previous reports (6). The transcript levels of *adhE2*, *thlA*, *crt*, *bcd*, and *hbd* genes in the *rex*-inactivated mutant were not significantly affected by MV addition and were higher than the levels measured in MV-exposed wild-type cells (Fig. 6A). These results strongly suggest that Rex responds to the increase in intracellular NADH/NAD^+ ratio achieved by MV exposure, leading to derepression of Rex target genes. The *ptb*, *buk*, and *ldh* genes in the wild type were not induced when MV was present in the medium (Fig. 6A), although these genes were identified as Rex direct target genes. This may be explained by possible involvement in their regulation of other, still-unknown regulatory mechanisms in the presence of MV.

On the other hand, exposure to H_2O_2 resulted in 5- to 20-fold-reduced mRNA levels of Rex regulon members *adhE2*, *ldh*, *thlA*,

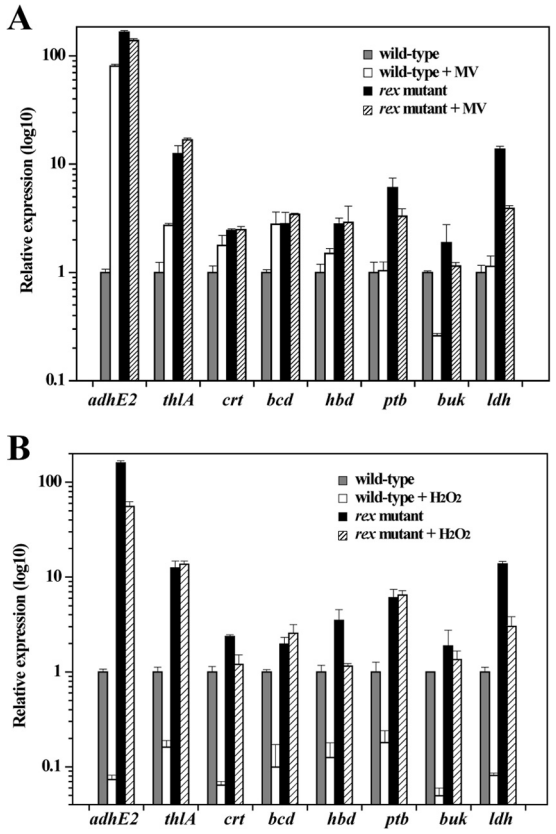

FIG 6 Effect of exposure to MV (A) or H_2O_2 (B) on transcript levels of the genes involved in fermentation in *C. acetobutylicum* wild-type and *rex*-inactivated mutant strains. The strains were grown in P2 minimal medium without or with addition of 1 mM MV or 30 μM H_2O_2. Total RNA was isolated from cells harvested in the exponential growth phase at an OD_{600} of about 2.0. The mRNA levels of each gene were determined by qRT-PCR and normalized to the gene expression in the wild-type strain grown in the absence of MV or H_2O_2. Data represent means ± standard deviations of values from six measurements starting from two independent cultures. Differences in the mRNA levels of *adhE2*, *thlA*, *crt*, *bcd*, and *hbd* genes in the wild type between the absence and presence of MV are statistically significant ($P < 0.01$), while the mRNA levels of all the studied genes in the wild type are significantly different ($P < 0.01$) upon exposure to H_2O_2.

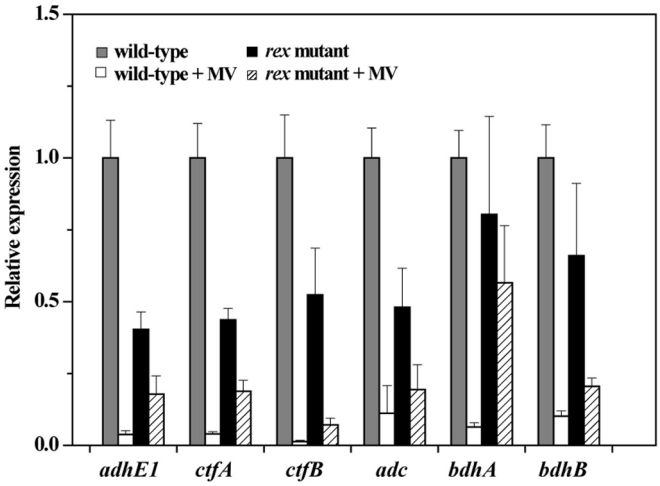

FIG 7 Effect of *rex* inactivation and MV addition on transcript levels of the fermentation genes that are not members of the predicted Rex regulon. Data represent means ± standard deviations of values from six measurements starting from two independent cultures and are normalized to the expression level in the wild type without MV exposure. Differences in the mRNA levels of *adhE1*, *ctfA*, *ctfB*, and *adc* genes between the wild type and *rex*-inactivated mutant are statistically significant ($P < 0.01$), while the mRNA levels of all the studied genes in the wild type are significantly different ($P < 0.01$) upon treatment with MV.

crt, *bcd*, *hbd*, *ptb*, and *buk* in the wild type (Fig. 6B). These genes in the *rex*-inactivated mutant showed unaltered or 2- to 4-fold-decreased expression levels in the presence of H_2O_2 compared to the culture without H_2O_2 addition. Thus, the effect of H_2O_2 addition on expression of these genes in the *rex*-inactivated mutant was much smaller than that in the wild type. These results indicate that Rex represses its target genes in response to the decrease in intracellular NADH/NAD^+ ratio achieved by H_2O_2 exposure. Therefore, transcriptional analyses of *C. acetobutylicum* wild type and *rex*-inactivated mutant exposed to MV or H_2O_2 reveal that Rex monitors the NADH/NAD^+ ratio *in vivo* to regulate expression of genes in its regulon.

In addition, we studied the effect of *rex* inactivation on the expression of central metabolic genes that are not members of the predicted Rex regulon (Fig. 7). They include the *adhE1* gene encoding bifunctional alcohol/acetaldehyde dehydrogenase; *ctfA*, *ctfB*, and *adc* genes responsible for acetone formation; and *bdhA* and *bdhB* genes encoding butanol dehydrogenases. Expression of these genes in the wild type was significantly downregulated by MV addition, which largely coincides with previous reports (6). The transcript levels of *adhE1*, *ctfA*, *ctfB*, and *adc* genes in the *rex*-inactivated mutant were about 2-fold lower than those in the wild type, and expression of *bdhA* and *bdhB* genes was not significantly affected by *rex* mutation in the absence of MV. Rex proteins are known to be transcriptional repressors in other bacterial species, and no binding of *C. acetobutylicum* Rex was observed for the promoter regions of the *adhE1-ctfAB* operon and *adc*, *bdhA*, and *bdhB* genes in EMSAs (data not shown), suggesting that Rex may indirectly regulate the expression of the *adhE1-ctfAB* operon and the *adc* gene in *C. acetobutylicum*.

Rex modulates fermentation product formation and oxidative stress tolerance in *C. acetobutylicum*. To elucidate the role of Rex in regulation of central metabolism in *C. acetobutylicum*, we compared the effects of MV exposure on fermentation product formation between the wild-type and *rex*-inactivated mutant strains. As shown in Fig. 8, the *rex*-inactivated mutant grew more slowly than the wild type in the presence of MV, whereas the growth rates of the two strains were similar in cultures without MV addition. Determination of fermentation product formation revealed different product spectra between the wild type and *rex*-inactivated mutant in the absence of MV (Fig. 8), which is in accordance with a recent report (10). Mutation of *rex* resulted in a significantly increased ethanol and a slightly elevated butanol production, while acetone synthesis was reduced; thus, the alcohol (butanol plus ethanol)-to-acetone ratio was improved from 2.7 to 4.8 (Fig. 8). Complementation of the *rex*-inactivated mutant by using a plasmid constitutively expressing *rex* restored a typical wild-type fermentation profile (see Fig. S3 in the supplemental material). Alcohol formation of the wild type was elevated by 22%, whereas acetone production was reduced 2.5-fold, by MV addition. In contrast, exposure of the *rex*-inactivated mutant to MV

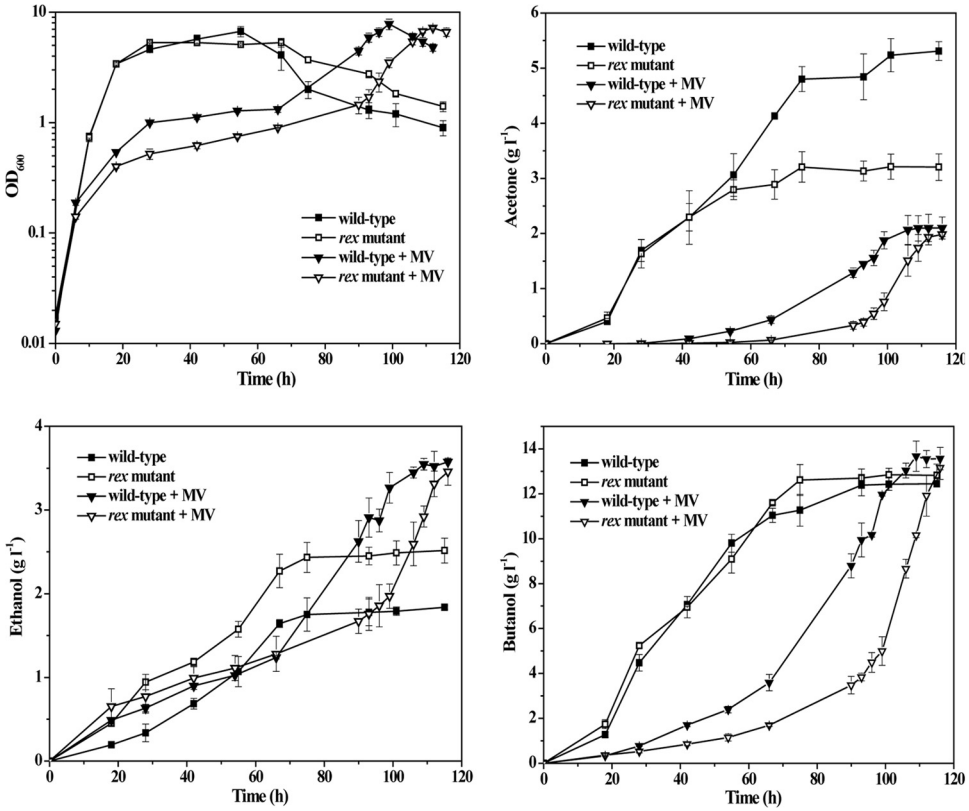

FIG 8 Cell growth and fermentation product formation in batch cultures of *C. acetobutylicum* wild-type and *rex*-inactivated mutant strains without or with addition of MV. The strains were grown in P2 minimal medium containing 60 g liter^{-1} of glucose. At an OD_{600} of about 0.15, MV was added to a final concentration of 1 mM. Cell growth was monitored spectrophotometrically at 600 nm. Formation of acetone, butanol, and ethanol was determined by gas chromatography. The data points and error bars represent means ± standard deviations of values from three independent cultures.

resulted in only marginally increased alcohol synthesis and 1.6-fold-decreased acetone production (Fig. 8). Therefore, the effect of MV addition on fermentation production formation in the wild type was more profound than in the *rex*-inactivated mutant. These results indicate that Rex modulates fermentation product formation and plays an important role in improving the alcohol-to-acetone ratio in *C. acetobutylicum* cultures.

Given that the *rex*-inactivated mutant exhibited a sharper decrease in intracellular NADH/NAD$^+$ ratio in response to H_2O_2 exposure than the wild type (Table 3), we wondered whether Rex deficiency would influence the capability of *C. acetobutylicum* to cope with oxidative stress. Hydrogen peroxide killing assays were used to assess the impact of *rex* mutation on oxidative stress tolerance of *C. acetobutylicum*. The wild-type and *rex*-inactivated mutant strains were incubated with an iron chelator (1 mM dipyridyl) and 50, 100, or 200 μM H_2O_2 for 30 min, and the survival of cells was determined as CFU. Results showed that the *rex*-inactivated mutant was more sensitive to H_2O_2 than the wild type (Fig. 9). The survival rate of the *rex*-inactivated mutant was approximately 2.5-fold lower than that of the wild type in the presence of 100 and 200 μM H_2O_2. Expression of a plasmid-encoded *rex* from a constitutive promoter in the *rex*-inactivated mutant restored a wild-type tolerance to H_2O_2. To understand why Rex deficiency increases susceptibility to oxidative stress, we compared between the wild-type and *rex*-inactivated mutant strains the expression levels of the genes encoding the components involved in detoxification. They include reverse rubrerythrins (*rbr3A-rbr3B*), desulfoferrodoxin (*dfx*), rubredoxin (*rd*), NADH-dependent rubredoxin oxidoreductase (*nror*), and the oxygen-reducing flavodiiron proteins (*fprA1* and *fprA2*). As shown in Fig. 10, the expression levels of these genes were decreased 2- to

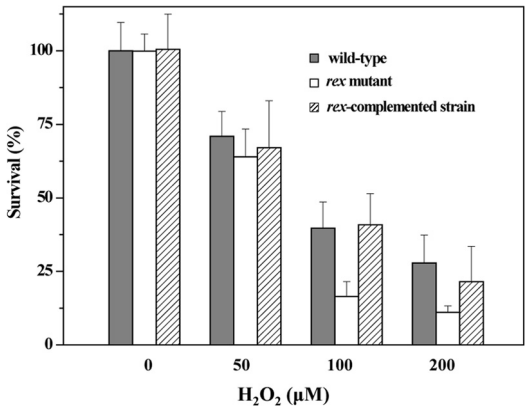

FIG 9 Survival of *C. acetobutylicum* wild-type, *rex*-inactivated mutant, and *rex*-complemented strains after hydrogen peroxide treatment. The strains were grown in P2 minimal medium to an OD_{600} of about 2.0. Then, cells were exposed to 1 mM iron chelator 2,2′-dipyridyl and the indicated concentrations of H_2O_2 or the equal volume of H_2O. After incubation at 37°C for 30 min, the CFU were determined as the survival of cells and normalized to the number obtained for the nonstressed wild type. Data represent means ± standard deviations of values from three independent experiments.

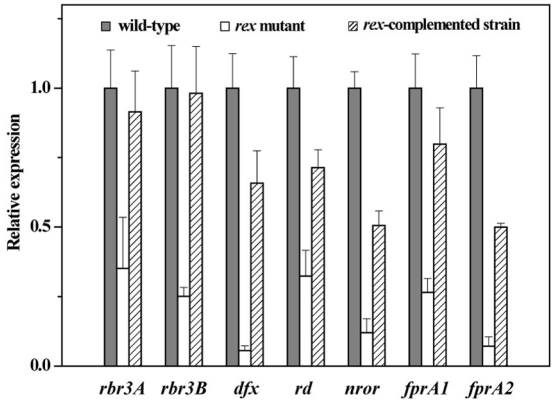

FIG 10 Influence of *rex* inactivation on transcript levels of the genes involved in detoxification of ROS and molecular O_2 in *C. acetobutylicum*. Total RNA was isolated from the wild-type, *rex*-inactivated mutant, and *rex*-complemented strains grown in P2 minimal medium and harvested at an OD_{600} of about 2.0. The expression levels of each gene were normalized to the gene expression in the wild-type strain. Data represent means ± standard deviations of values from six measurements starting from two independent cultures. Differences in the mRNA levels of all the studied genes between the wild type and *rex*-inactivated mutant are statistically significant ($P < 0.01$).

18-fold in the *rex*-inactivated mutant compared to those in the wild type. Following constitutive expression of a plasmid-borne *rex* in the *rex*-inactivated mutant, the transcription of these genes was largely restored. These genes are not preceded by a candidate Rex-binding site, suggesting an indirect effect of Rex on their activation. These results indicate that Rex is involved in regulation of oxidative stress response in *C. acetobutylicum*.

DISCUSSION

In this work, we performed comparative genomic reconstruction of Rex regulons in 11 diverse clostridial species by combining the identification of candidate Rex-binding sites with cross-genomic comparison of regulons. Considerable variations were revealed in the sizes and gene contents of reconstructed Rex regulons between different species. The predicted Rex-binding sites in the genomes of *Clostridium* spp. were experimentally validated. New target genes of Rex in *C. acetobutylicum*, which are involved in fermentation, NAD biosynthesis, and sulfite reduction, were identified. Moreover, we compared the effects of exposure to methyl viologen or H_2O_2 on intracellular NADH/NAD^+ ratio, expression of Rex targets, and physiology between *C. acetobutylicum* wild-type and *rex*-inactivated mutant strains. Our results demonstrate that Rex responds to changes in the NADH/NAD^+ ratio *in vivo* to regulate gene expression and modulates fermentation product formation and oxidative stress response in *C. acetobutylicum*.

Addition of MV to cultures is one of the approaches that have been widely used to shift the metabolism of *C. acetobutylicum* away from hydrogen production toward alcohol formation (5). Under this condition, the intracellular NADH/NAD^+ ratio increases; thus, Rex dissociates from its operator sites, leading to derepression of *adhE2*, *thlA*, *crt*, *bcd*, and *hbd* genes. Among the four known genes encoding alcohol or butanol dehydrogenases (i.e., *adhE1*, *adhE2*, *bdhA*, and *bdhB*), only the *adhE2* gene is a direct target of Rex and upregulated by MV addition (Fig. 6 and 7). Consistently, previous studies have shown that the *adhE2*-encoded NADH-dependent aldehyde/alcohol dehydrogenase is related to an alcohologenic phenotype (41). Therefore, Rex-mediated regulation of *adhE2*, *thlA*, *crt*, *bcd*, and *hbd* genes probably plays a crucial role in enhanced alcohol production in MV-exposed *C. acetobutylicum*. In fact, we found that although MV addition resulted in a remarkable increase in NADH/NAD^+ ratio in the *rex*-inactivated mutant (Table 3), it did not significantly affect the expression of *adhE2*, *thlA*, *crt*, *bcd*, and *hbd* genes and its influence on fermentation product formation in the *rex*-inactivated mutant was modest compared to that in the wild type (Fig. 6 and 8). The *rex*-inactivated mutant exhibited a notably reduced acetone production, which is consistent with the significantly decreased transcript levels of *ctfA*, *ctfB*, and *adc* genes (Fig. 7 and 8). This result suggests that Rex may modulate acetone formation in *C. acetobutylicum* by regulating expression of the acetone synthesis genes, although *ctfA*, *ctfB*, and *adc* genes are not Rex direct targets. Our speculation is that an indirect effect of Rex might occur via additional regulators such as Spo0A, which is a major regulator of sporulation and required for transcription of *ctfA*, *ctfB*, and *adc* genes in *C. acetobutylicum* (42, 43), which is consistent with the observation of impaired spore formation for the *rex*-inactivated mutant (data not shown). Therefore, our results reveal that Rex plays an important role in improving the alcohol-to-acetone ratio in *C. acetobutylicum* cultures.

In addition to MV addition, other approaches such as carbon monoxide sparging or utilization of glycerol as a substrate have also been used to shift the solvent ratio toward butanol in *C. acetobutylicum* cultures. These approaches aim to inhibit hydrogenase activity, and the reduction of hydrogen formation results in an increased electron flow toward butanol synthesis. We speculate that Rex-dependent regulation in response to intracellular NADH/NAD^+ ratio is also involved in these physiological interventions. Genetic manipulations have also been applied to reduce by-product formation of *C. acetobutylicum*; however, most of these attempts did not result in a desired butanol producer (44, 45). For example, Jiang et al. constructed an *adc*-inactivated mutant which produced much less acetone, but butanol titers were also reduced and could be restored to the level of the parent strain only with pH control and MV addition to cultures (45). Based on understanding of redox-dependent regulatory mechanisms, alternative engineering targets could be designed to alter the intracellular redox status and improve the butanol production of *C. acetobutylicum*.

The strictly anaerobic clostridia can withstand limited air exposure upon activation of their reductive machinery for the scavenging of ROS and molecular O_2. We found that the *C. acetobutylicum rex*-inactivated mutant is more susceptible to H_2O_2 killing than the wild type (Fig. 9), indicating that Rex modulates oxidative stress tolerance in this obligate anaerobe. Although involvement of Rex in regulation of the oxidative stress response has also been reported for the facultative anaerobe *S. mutans* (15), the mechanisms may be different between *S. mutans* and clostridia. Our results demonstrate that Rex responds to the decrease in intracellular NADH/NAD^+ ratio achieved by H_2O_2 exposure to repress its target genes, including those encoding NADH-consuming enzymes in central metabolism (e.g., *ldh*, *adhE2*, *bcd-etfBA*, and *hbd*) (Fig. 6). This may increase the availability of reducing power needed for reduction of H_2O_2. Moreover, expression of the genes encoding the components involved in detoxification of ROS and oxygen was downregulated in the *rex*-inactivated mutant (Fig. 10), although these genes are not preceded by Rex-binding sites,

suggesting that Rex could indirectly enhance the detoxification system in *C. acetobutylicum*. Because these genes are primary targets of the transcriptional repressor PerR in *C. acetobutylicum* according to a previous study (46), we speculate that Rex may regulate expression of these genes via PerR or other transcription factors. However, more work is needed to elucidate the mechanism of the involvement of Rex in regulation of the oxidative stress response. The Rex and PerR regulatory systems that both are widely distributed in *Clostridium* species seem to play important roles in the oxidative stress defense in *C. acetobutylicum*, but they sense different signals and possess different direct targets. Whereas Rex senses intracellular NADH/NAD^+ ratio to regulate many fermentation genes, PerR is a peroxide sensor that negatively controls expression of the genes involved in the oxygen and ROS detoxification.

An important role of Rex in maintaining NADH/NAD^+ homeostasis in *C. acetobutylicum* was revealed based on our measurements of intracellular NADH and NAD^+ concentrations. When exposed to MV or H_2O_2, the *rex*-inactivated mutant exhibited larger fluctuations in the NADH/NAD^+ ratio than the wild type (Table 3). Further studies are required to identify the mechanism by which Rex functions to prevent large fluctuations in the NADH/NAD^+ ratio in *C. acetobutylicum*. It is hypothesized that Rex regulates the expression of NADH-consuming enzymes (e.g., aldehyde/alcohol dehydrogenase) in response to increased NAD(P)H availability or oxidative stress to help maintain redox homeostasis in the cell. In addition to the direct and indirect targets identified in this study, Rex may also control the expression of many other enzymes involved in the redox balance in *C. acetobutylicum*, and the transcriptome analysis of the *rex*-inactivated mutant is now under way. It is worth noting that the influence of exposure to MV or H_2O_2 on intracellular NAD^+ concentration was more profound than that on NADH concentration (Table 3). This suggests that NAD^+ has an important role in modulating the DNA-binding activity of Rex in *C. acetobutylicum*, although *in vitro* binding assays showed that the binding affinity of Rex for NAD^+ is much lower than that for NADH (Fig. 5) (16). NAD^+ competes with NADH for binding to Rex, thereby impairing the inhibitory effect of NADH on Rex-DNA complex formation. Allosteric activation for DNA binding by NAD^+ has been reported for *B. subtilis* Rex (16) and may also exist for *C. acetobutylicum* Rex.

This study gains an insight into the potential regulatory role of Rex in clostridial species other than *C. acetobutylicum* based on comparative genomic reconstruction of Rex regulons. In *C. beijerinckii*, another solvent-producing species, the predicted Rex regulon contains genes involved in fermentation (*pflBA*, *adhA*, *adhA2*, *thlA*, *thlA2*, *crt-bcd-etfBA-hbd*, *ptb-buk*, *butA*, and *fld*-Cbei_4318) and hydrogen production (*hydB*). Experimental evidence that Rex binds upstream of these target genes in *C. beijerinckii* was provided by EMSAs (Fig. 3). Among these candidate Rex targets, the *adhA* (Cbei_2181) and *adhA2* (Cbei_1722) genes encode two primary alcohol dehydrogenases responsible for production of butanol and ethanol in *C. beijerinckii* (47). The *hydB* gene (Cbei_0327) codes for a hydrogenase that uses reduced ferredoxin as the electron donor, and reduced ferredoxin could be generated by pyruvate ferredoxin/flavodoxin oxidoreductase encoded by Cbei_4318. The predicted regulation of both *hydB* and Cbei_4318 expression by Rex suggests that Rex may be involved in modulation of hydrogen production in *C. beijerinckii*. Consistently, previous studies have shown that the presence of reduced electron shuttling compounds such as anthrahydroquinone-2,6-disulfonate increased the hydrogen yield of *C. beijerinckii*, suggesting that hydrogen production is modulated by the redox status in the cell (48). To assess the regulatory role of Rex in *C. beijerinckii*, we constructed the *rex*-inactivated mutant of *C. beijerinckii*. Our results showed that *rex* inactivation did not significantly alter the fermentation product spectra in *C. beijerinckii*, although it resulted in derepression of predicted Rex target genes (see Fig. S4 in the supplemental material). One possible explanation is that Rex coordinately regulates alcohol formation and hydrogen production and the distribution of electron flow through these pathways is generally rigid in *C. beijerinckii*. So far, manipulation of the redox balance to shift the electron flow away from hydrogen production toward alcohol production is limited to *C. acetobutylicum*, and its successful application in *C. beijerinckii* has never been reported. Therefore, the redox-dependent regulatory mechanisms in *C. beijerinckii* probably differ from that in *C. acetobutylicum*. The different metabolic responses to perturbations in cellular redox balance between the two solventogenic clostridia may be partly attributed to the variability of the Rex regulon members.

The acetogenic *C. carboxidivorans*, *C. ljungdahlii*, and *Clostridium autoethanogenum* are capable of using the Wood-Ljungdahl pathway to fix CO_2 or CO and convert it into acetyl-CoA. This feature makes them become promising production strains for industrial syngas fermentations (49). However, regulation of the Wood-Ljungdahl pathway genes in these acetogenic clostridia remains to be explored. Here, we predicted a candidate Rex-binding site located upstream of the Wood-Ljungdahl pathway gene cluster in the genomes of *C. carboxidivorans* and *C. ljungdahlii*. Binding of Rex to the promoter region of this gene cluster in both clostridia was verified by EMSAs (Fig. 3). A putative Rex-binding site upstream of the Wood-Ljungdahl pathway gene cluster was also identified in the genome of *C. autoethanogenum* (data not shown). This suggests that Rex may play a role in regulation of CO or CO_2 reduction in *C. carboxidivorans*, *C. ljungdahlii*, and *C. autoethanogenum*. Whether *rex* inactivation will lead to derepression of Wood-Ljungdahl pathway genes and improvement of syngas fermentation in these acetogenic clostridia needs to be tested. Nevertheless, this study offers an insight into redox-dependent gene regulation in these species, which could be useful for designing sophisticated metabolic engineering approaches to increase the product yields of syngas fermentation.

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