Redox-responsive repressor Rex modulates alcohol production and oxidative stress tolerance in *Clostridium acetobutylicum*.

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Abstract

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, tricarboxylic acid cycle, NAD biosynthesis, nitrate and sulphite 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 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\(_2\)O\(_2\) 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.
INTRODUCTION

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 re-oxidized 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 towards 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, rubrerythrins (8). Clostridia use their reducing equivalents to reduce the toxic reactive oxygen species (ROS) and molecular O₂, 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 influence H$_2$O$_2$ 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 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 acids-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 sulphite 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$_2$O$_2$ 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 BLASP 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 web-server (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 are high scored and/or conserved in two or more genomes were included in 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 was 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<sub>600</sub>; ~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<sub>2</sub>HPO<sub>4</sub>, 0.5 g of KH<sub>2</sub>PO<sub>4</sub>, 2.2 g of CH<sub>3</sub>COONH<sub>4</sub>, 0.2 g of MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.01 g of MnSO<sub>4</sub>·H<sub>2</sub>O, 0.01 g of NaCl, 0.01 g of FeSO<sub>4</sub>·7H<sub>2</sub>O, 1 mg of p-aminobenzoic, 1 mg of vitamin B<sub>1</sub>, 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<sub>600</sub> of about 0.15. For hydrogen peroxide challenge experiments, cells were grown in P2 minimal medium to an OD<sub>600</sub> of about 2.0. Then cells were exposed to 50, 100, 200 μM H<sub>2</sub>O<sub>2</sub> or the equal volume of H<sub>2</sub>O. Because the sensitivity of C. acetobutylicum
towards H$_2$O$_2$ was largely dependent on Fenton chemistry (28). 1 mM of the 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 survived cells was determined as described previously (29). Briefly, aliquots of appropriate dilutions were plated on CGM medium and incubated anaerobically for 36 h at 37°C. The colony-forming units 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 TargeTron™ gene knockout system (Sigma). The PCR product was digested and ligated to a targetron vector pWJ1 (31), yielding the plasmid pWJ1-*rex*. The plasmid was methylated *in vivo* in *E. coli* ER2275 (pAN1) (32) and electroporated into *C. acetobutylicum* ATCC 824. The transformants were selected on 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. 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$_2$O$_2$. 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). 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. 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 lysine residue, was constructed with two steps of PCR using pET28a-rex as template and the mutagenic primers and flanking primers (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 OD₆₀₀ 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.** The 180-bp DNA fragments in the promoter region of individual genes were PCR-amplified using the primers shown in Table S1. 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 DTT, 10 mM MgCl₂, 5% glycerol, 0.8 µg bovine serum albumin (BSA), and 1 µg salmon sperm DNA (non-specific 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 (FujiFlim, Japan). For the determination of apparent dissociation constants (Kₐ), 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 Kₐ 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 non-fluorescent detection reagent that is reduced in the presence of NADH to produce its fluorescent analog. Briefly, cells were harvested at mid-exponential-growth phase ($\text{OD}_{600}$ of about 2.0) by centrifuging 2 ml of culture broth at 9000 x 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 non-fluorescent detection reagent to form NAD$^+$ and the fluorescent analog. The concentration of the formed fluorescent analog was then determined at 550-nm excitation, 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 $\text{OD}_{600}$ and a previously reported intracellular aqueous volume of 1.67 $\mu$l 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 Clostridiales (24), which has consensus TTGTAANNNTTAACAA, 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 co-regulated 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 adhA gene encoding alcohol dehydrogenase, adhE2 gene encoding bifunctional alcohol/acetaldehyde dehydrogenase, thlA
gene and \textit{crt-bcd-etfBA-hbd} operon responsible for the conversion of acetyl-CoA to butyryl-CoA, and \textit{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 (\textit{ldh, pflBA, ctfAB, butA, pfor}), hydrogen production (\textit{hydB}), TCA cycle (\textit{frd, maeB}), and nitrate and sulfite reduction (\textit{narK, asrABC, asrT}). In addition, the NAD biosynthetic genes \textit{nadABC} were identified as candidate members of Rex regulon in \textit{C. acetobutylicum}. Regulation of the Wood-Ljungdahl pathway (\textit{codH-cooC-flhs-fchA-folD-metF-lpdA-cooC-acsDCEB}) by Rex, which is used to fix CO\textsubscript{2} or CO, was predicted for \textit{C. carboxidivorans} and \textit{C. ljungdahlii}.

In summary, the comparative genomics analysis allowed us to reconstruct the Rex regulons in 11 diverse clostridial species. Among these species, \textit{C. acetobutylicum} has one of the largest set of Rex targets, including 17 genes organized in 7 operons that contain not only the known targets (\textit{ldh, adhE2, thlA, crt-bcd-etfBA-hbd}) but also the newly identified members (\textit{ptb-buk, nadABC, 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 \textit{C. acetobutylicum} as described below.

\textbf{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 \textit{C. acetobutylicum}, which was overexpressed in \textit{E. coli} with the N-terminal His\textsubscript{6} tag and purified with a nickel-chelating affinity column. For all predicted Rex target operons in \textit{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 3000 nM of 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 non-specific 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*ₐ) values of Rex protein interacting the tested *C. acetobutylicum* DNA fragments were determined, which varied in a wide range from 23 nM to 393 nM (Fig. 2). According to the *K*ₐ values, the tested DNA fragments can be divided into two groups. For the first group including the promoter fragments of *adhE2*, *ldh* genes, and *crt-bcd-etfBA-hbd* operon, Rex protein exhibited a high affinity and the *K*ₐ values were in the range of 23 to 37 nM. The second group includes the fragments from the promoter regions of *thlA* gene, *asrTABC*, *ptb-buk*, and *nadABC* operons. The *K*ₐ 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-pfor*, *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*, *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 3B). For the *C. carboxidivorans narK* fragment, two shifted Rex-DNA complexes were detected, supporting our prediction that two DNA binding sites are present (Table S2). Most of the promoter
fragments were completely shifted with 1000 nM Rex (Fig. 3A and 3B). In contrast, the promoter fragments of C. beijerinckii ldh gene and C. carboxidivorans crt-hbd-thlA-bcd-etfBA operon, which do not contain predicted Rex-binding sites, were not shifted even with 3000 nM Rex protein (Fig. 3C).

**Characterization of the Rex-binding motif in clostridia.** The identified clostridial Rex-binding motif has consensus TTGTTAANNNNTTAACAA, which deviates in two positions (i.e. positions 5 and 14) from the common consensus TTGTGAANNNNTTCACAA 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 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 substituted the thymine at position 5 to guanine or/and 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 thymine5 or adenine14 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 thymine5 and adenine14 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 guanine5 of the DNA operator according to a structural study of T. aquaticus Rex (17). However, the Lys47 residue was substituted to glutamine (Gln51) in the Rex proteins from Clostridium spp. (Fig. S1). To assess if this residue substitution influences the Rex-DNA contacts, the Gln51 residue of C. acetobutylicum Rex
was exchanged to 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 *crt-bcd-ettBA-hbd* operon. Nevertheless, when the thymine5 or adenine14 in the binding sequence were substituted 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 with the thymine5 and adenine14 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 *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 to 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 to 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 varying concentrations of NADH and NAD⁺, which covers 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 RT-PCR. The two strains were cultivated in minimal medium with 60 g l$^{-1}$ of glucose as carbon source, and no differences in cell growth were observed for them. For comparison of transcript levels, cells were 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 $\geq 160$-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 (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 1.5–3-fold elevated transcript level in the *rex*-inactivated mutant. Expression of the *ptb-buk* operon was also increased by *rex* mutation. Therefore, the quantitative RT-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 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\(_2\)O\(_2\)). 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 \(\mu\)M H\(_2\)O\(_2\) 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 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\(^+\) ratio was 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. On the contrary, the intracellular NADH/NAD\(^+\) ratio was decreased by 21% and 56% in the wild-type and *rex*-inactivated mutant, respectively, by H\(_2\)O\(_2\) addition (Table 3). Therefore, MV addition resulted in a remarkable increase in intracellular NADH/NAD\(^+\) ratio, whereas exposure to H\(_2\)O\(_2\) significantly reduced the NADH/NAD\(^+\) ratio. The *rex*-inactivated mutant showed larger fluctuations in the NADH/NAD\(^+\) ratio than the wild-type when exposed to MV or H\(_2\)O\(_2\), suggesting that Rex plays an important role in maintaining NADH/NAD\(^+\) homeostasis in ***C. acetobutylicum***.

**Rex monitors NADH/NAD\(^+\) ratio in vivo to regulate gene expression.** The effect of exposure to MV or H\(_2\)O\(_2\) on expression of genes in 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 quantitative RT-PCR, because the
fermentation genes comprise the major direct targets of clostridial Rex. As shown in Fig. 6A,
expression of Rex target genes \textit{adhE2}, \textit{thlA}, \textit{crt}, \textit{bcd}, and \textit{hbd} in the wild-type was
significantly up-regulated by MV addition. Most strikingly, the \textit{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 \textit{adhE2}, \textit{thlA}, \textit{crt}, \textit{bcd}, and \textit{hbd} genes in the \textit{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 \textit{ptb}, \textit{buk}, and \textit{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$_2$O$_2$ resulted in 5–20-fold reduced mRNA levels of Rex
regulon members \textit{adhE2}, \textit{ldh}, \textit{thlA}, \textit{crt}, \textit{bcd}, \textit{hbd}, \textit{ptb}, and \textit{buk} in the wild-type (Fig. 6B).
These genes in the \textit{rex}-inactivated mutant showed unaltered or 2–4-fold decreased expression
levels in the presence of H$_2$O$_2$ compared to the culture without H$_2$O$_2$ addition. Thus the effect
of H$_2$O$_2$ addition on expression of these genes in the \textit{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$_2$O$_2$ exposure.
Therefore, transcriptional analyses of \textit{C. acetobutylicum} wild-type and \textit{rex}-inactivated mutant
exposed to MV or H$_2$O$_2$ reveal that Rex monitors NADH/NAD$^+$ ratio \textit{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 *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 down-regulated 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 *adhE1-ctfAB* operon, *adc*, *bdhA*, and *bdhB* genes in EMSAs (data not shown), suggesting that Rex may indirectly regulate the expression of *adhE1-ctfAB* operon and *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 effect 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 rate of both strains was 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 (Fig. S3). Alcohol formation of the wild-type was elevated by 22%, whereas acetone production was reduced 2.5-fold, by MV addition. By contrast, exposure of the rex-inactivated mutant to MV 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 H₂O₂ 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 H₂O₂ for 30 min, and the survival of cells was determined as colony forming units. Results showed that the rex-inactivated mutant was more sensitive to H₂O₂ 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 H₂O₂. Expression of a plasmid-encoded rex from a constitutive promoter in the rex-inactivated mutant restored a wild-type tolerance to H₂O₂. 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 ruberythmins (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 2–18-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 size and gene content 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 H2O2 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 towards 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 up-
regulated 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 indirect effect of Rex 
might occur via additional regulators such as Spo0A that 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 towards 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 ade-inactivated mutant which produced much less acetone, but butanol titres were also reduced and could only be restored to the level of the parent strain 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 its reductive machinery for the scavenging of ROS and molecular O$_2$. We found that *C. acetobutylicum* rex-inactivated mutant is more susceptible to H$_2$O$_2$ 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 oxidative stress response has also been reported for facultative anaerobe *S. mutans* (15), the mechanism 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$_2$O$_2$ 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 availability of reducing power needed for reduction of H$_2$O$_2$. Moreover, expression of the genes encoding the components involved in detoxification of ROS and oxygen was down-regulated 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 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 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 defence 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$_2$O$_2$, 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 how 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$_2$O$_2$ 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-pfor*) 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 the *pfor* gene (*Cbei_4318*). The predicted regulation of both *hydB* and *pfor* 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 (Fig. S4 in the supplemental material). One possible explanation is that Rex may coordinateely regulate 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 towards 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 CO₂ 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 CO₂ 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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disruption by use of a group II intron (targetron) vector in Clostridium

Confirmation and elimination of xylose metabolism bottlenecks in glucose
phosphoenolpyruvate-dependent phosphotransferase system-deficient Clostridium


**TABLE 1** Rex regulons in 11 species of clostridia*.

<table>
<thead>
<tr>
<th>Operon and metabolism</th>
<th>Clostridium species</th>
<th>Functional role</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fermentation</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>ldh</em></td>
<td>+* − + − − − + − 0 0 0 0</td>
<td>t-Lactate dehydrogenase</td>
</tr>
<tr>
<td><em>pfBA</em></td>
<td>− +* − − − − + 0 + 0 0</td>
<td>Pyruvate formate-lyase</td>
</tr>
<tr>
<td><em>adhA</em></td>
<td>0 +* + + − + + + + + +</td>
<td>Alcohol dehydrogenase [Fe]</td>
</tr>
<tr>
<td><em>adhE2</em></td>
<td>+* − + + − 0 + + + + + −</td>
<td>Alcohol/acetaldehyde dehydrogenase</td>
</tr>
<tr>
<td><em>thlA</em></td>
<td>+* + + + + + + + − 0</td>
<td>Acetyl-CoA acetyltransferase</td>
</tr>
<tr>
<td><em>crt-bcd-etfBA-hbd</em></td>
<td>+* + + + + + + + + − 0</td>
<td>Butyryl-CoA synthesis enzymes</td>
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<tr>
<td><em>ctfAB</em></td>
<td>− − − 0 0 +* 0 0 0 0 0</td>
<td>CoA-transferase</td>
</tr>
<tr>
<td><em>pht-buk</em></td>
<td>+* + − + 0 0 + + − + 0</td>
<td>Phosphotransbutyrylase, butyrate kinase</td>
</tr>
<tr>
<td><em>butA</em></td>
<td>0 +* + + 0 − 0 0 0 − −</td>
<td>2,3-Butanediol dehydrogenase</td>
</tr>
<tr>
<td><em>hydB</em></td>
<td>0 +* − − 0 + − − 0 − −</td>
<td>Fe-hydrogenase</td>
</tr>
<tr>
<td><em>fdd-pfor</em></td>
<td>− +* − + − − − − 0 0 0</td>
<td>Flavodoxin, pyruvate flavodoxin/ferredoxin oxidoreductase</td>
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<tr>
<td>TCA cycle</td>
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<td></td>
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<tr>
<td><em>frdA</em></td>
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<td>Fumarate reductase flavoprotein subunit</td>
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<tr>
<td><em>maeB</em></td>
<td>0 − +* + 0 0 0 0 0 − −</td>
<td>Malic enzyme</td>
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<tr>
<td>NAD biosynthesis</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>nadABC</em></td>
<td>+* − − − − − − 0 − − −</td>
<td>NAD biosynthesis enzymes</td>
</tr>
<tr>
<td>Nitrate and sulfate reduction</td>
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<tr>
<td><em>natAB</em></td>
<td>0 0 0 + 0 + 0 + 0 +* 0 +</td>
<td>Nitrate reductase</td>
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<tr>
<td><em>natK</em></td>
<td>0 0 0 0 0 0 0 0 − 0 0 +* 0</td>
<td>Nitrate/nitrite transporter</td>
</tr>
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<td><em>asrABC</em></td>
<td>+* − − − 0 − − − − − + +</td>
<td>Sulfite reductase</td>
</tr>
<tr>
<td><em>asrT</em></td>
<td>+* − − 0 0 0 − − − − + 0</td>
<td>Predicted sulfite/sulfate transporter</td>
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<td>Wood-Ljungdahl pathway</td>
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<td></td>
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<td><em>codH-cooC-fhs-fchA</em></td>
<td>0 0 0 0 0 0 0 0 0 +* +*</td>
<td>Wood-Ljungdahl pathway enzymes</td>
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<tr>
<td>-fold-metF-lpdA*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-cooC-acsDCEB*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>gsdH</em></td>
<td>0 0 0 0 0 0 0 0 0 +* +</td>
<td>Betaine/glycine reductase</td>
</tr>
<tr>
<td><em>nosE</em></td>
<td>0 − + − − 0 + +* + + 0 0 0</td>
<td>NADH oxidase</td>
</tr>
<tr>
<td><em>bcd2-fldA-bcd-etfBA</em></td>
<td>0 0 0 0 0 0 0 0 0 0 +*</td>
<td>Acyl-CoA dehydrogenase, acyl-CoA transferase, flavoprotein</td>
</tr>
</tbody>
</table>

*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 asterisk. Genes without a candidate Rex binding site are indicated by −. The absence of orthologous gene(s) in the analyzed genomes is indicated by 0.
TABLE 2 Comparison of mRNA levels in *C. acetobutylicum* wild-type and rex-inactivated mutant using quantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene ID</th>
<th>mRNA ratio&lt;sup&gt;a&lt;/sup&gt; (rex mutant/WT)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>adhE2</em></td>
<td>CAP0035</td>
<td>164.75 ± 6.45</td>
</tr>
<tr>
<td><em>ldh</em></td>
<td>CAC0267</td>
<td>13.75 ± 0.84</td>
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<td><em>thlA</em></td>
<td>CAC2873</td>
<td>12.46 ± 2.34</td>
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<tr>
<td><em>crt</em></td>
<td>CAC2712</td>
<td>2.43 ± 0.10</td>
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<tr>
<td><em>bcd</em></td>
<td>CAC2711</td>
<td>2.81 ± 0.78</td>
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<tr>
<td><em>etfB</em></td>
<td>CAC2710</td>
<td>2.17 ± 0.20</td>
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<tr>
<td><em>etfA</em></td>
<td>CAC2709</td>
<td>1.55 ± 0.14</td>
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<tr>
<td><em>hbd</em></td>
<td>CAC2708</td>
<td>2.79 ± 0.37</td>
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<tr>
<td><em>ptb</em></td>
<td>CAC3076</td>
<td>6.07 ± 1.35</td>
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<tr>
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<td>CAC1025</td>
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<td>CAC1024</td>
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<td><em>nadC</em></td>
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<tr>
<td><em>asrT</em></td>
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<td><em>asrB</em></td>
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<td><em>asrC</em></td>
<td>CAC1515</td>
<td>1.69 ± 0.48</td>
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<sup>a</sup> Data represent means ± S.D. of values of mRNA ratios obtained from six measurements starting from two independent cultures. The strains were cultivated in the P2 minimal medium, and total RNA was isolated in the exponential growth phase at an OD<sub>600</sub> of about 2.0. The *p* value of the mRNA ratios for all the genes studied is smaller than 0.01.
**TABLE 3** Intracellular NADH and NAD$^+$ concentrations in *C. acetobutylicum* wild-type and *rex*-inactivated mutant\(^a\)

<table>
<thead>
<tr>
<th>Strains</th>
<th>Addition to cultures(^a)</th>
<th>NADH (mM)</th>
<th>NAD$^+$ (mM)</th>
<th>NADH/NAD$^+$</th>
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<tr>
<td>wild-type</td>
<td></td>
<td>0.19 ± 0.01</td>
<td>0.83 ± 0.01</td>
<td>0.23 ± 0.02</td>
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<tr>
<td>wild-type</td>
<td>MV</td>
<td>0.27 ± 0.03</td>
<td>0.46 ± 0.01</td>
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<td>wild-type</td>
<td>H$_2$O$_2$</td>
<td>0.20 ± 0.02</td>
<td>1.11 ± 0.11</td>
<td>0.18 ± 0.02</td>
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<tr>
<td><em>rex</em> mutant</td>
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<td>0.26 ± 0.02</td>
<td>1.02 ± 0.18</td>
<td>0.25 ± 0.02</td>
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<tr>
<td><em>rex</em> mutant</td>
<td>MV</td>
<td>0.34 ± 0.08</td>
<td>0.38 ± 0.07</td>
<td>0.92 ± 0.11</td>
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<tr>
<td><em>rex</em> mutant</td>
<td>H$_2$O$_2$</td>
<td>0.16 ± 0.02</td>
<td>1.44 ± 0.12</td>
<td>0.11 ± 0.02</td>
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</table>

\(^a\) Data represent means ± S.D. values 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$_2$O$_2$. The intracellular concentrations of NADH and NAD$^+$ were determined in the exponential growth phase at an OD$_{600}$ of about 2.0.
**FIGURE LEGENDS**

**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 black circles indicate the number of genomes where the target gene is preceded by a candidate Rex-binding site.

**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 *C. acetobutylicum* pflBA operon, which lacks a putative Rex binding site, was used.

**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 (lane 1) and in the presence of 60, 300, and 1000 nM of Rex protein (lanes 2 to 4). (B) EMSAs were performed in the absence (lane 1) and in the presence of 500, 1000, and 1500 nM of Rex protein (lanes 2 to 4). (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.

**FIG 4** Characterization of the Rex binding motif in clostridia. (A) Alignment of the Rex-binding sites in the promoter regions of *C. acetobutylicum* *crt-bcd-etfBA-hbd* operon, *ldh* gene, and *asrTABC* operon, *C. beijerinckii* *thlA* and *hydB* genes, *C. botulinum* *maeB* gene, and *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 *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 *crt* promoter and mutated fragments.

**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 (A) but with a range of physiological concentrations of NADH and NAD$^+$. 

**FIG 6** Effect of exposure to MV (A) or H$_2$O$_2$ (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 the P2 minimal medium without or with addition of 1 mM MV or 30 µM
H$_2$O$_2$. 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$_2$O$_2$. Data represent means ± S.D. 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$_2$O$_2$.

**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 ± S.D. 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.

**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 the P2 minimal medium containing 60 g l$^{-1}$ 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 ± S.D. of values from three independent cultures.
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 of the iron chelator 2,2’-dipyridyl and the indicated concentrations of H$_2$O$_2$ or the equal volume of H$_2$O. After incubation at 37°C for 30 min, the colony-forming units were determined as the survival of cells and normalized to the number obtained for the non-stressed wild-type. Data represent means ± S.D. 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 the 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 ± S.D. 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 ($p<0.01$).
Fig. 1.
**Fig. 2.**
Fig. 3.
Fig. 4.
### A

<table>
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<th>NADPH (μM)</th>
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Rex-DNA complex

Free DNA

### B

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Rex-DNA complex

Free DNA

Fig. 5.
Fig. 6.

A

B

Relative expression (log10)

adhE2 thlA crt bcd ptb buk hbd ldh

Wild-type
Wild-type + MV
rex mutant
rex mutant + MV

Relative expression (log10)

adhE2 thlA crt bcd ptb buk hbd ldh

Wild-type
Wild-type + H2O2
rex mutant
rex mutant + H2O2
Fig. 7.
Fig. 8.
Fig. 9. 

The graph shows the survival percentage (%) of three strains: wild-type, rex mutant, and rex-complemented strain, plotted against different concentrations of \( \text{H}_2\text{O}_2 \) in μM. The y-axis represents survival percentage, ranging from 0 to 100, and the x-axis represents \( \text{H}_2\text{O}_2 \) concentrations at 0, 50, 100, and 200 μM.
Fig. 10.