JB Accepts, published online ahead of print on 2 September 2014
J. Bacteriol. doi:10.1128/JB.02037-14

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

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Running Title: Redox-dependent gene regulation in clostridia

Key words: redox regulation; Rex; comparative genomics; clostridia

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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_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.

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 O_2, thereby protecting crucial oxygen-sensitive metabolic

64 enzymes (9). To generate the required reducing equivalents, the cellular redox balance needs
65 to be shifted accordingly.

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

80 Although the relative levels of NADH and NAD^+ have been shown to influence the
81 DNA-binding activity of Rex based on *in vitro* binding assays, it remains unclear whether
82 Rex monitors the NADH/NAD^+ ratio *in vivo* to control gene expression. Several genes
83 associated with fermentation pathways have been identified as Rex targets in *C.*
84 *acetobutylicum* (10). However, whether Rex also regulates transcription of other genes is not
85 known. The role of Rex-dependent regulation in *C. acetobutylicum* in response to an altered
86 cellular redox balance such as increased NAD(P)H availability or oxidative stress has not
87 been studied. Moreover, although Rex seems to be widely distributed in clostridia, little is
88 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. tetanii* 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_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 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_{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, 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, 200 μM H_2O_2 or the equal volume of H_2O. Because the sensitivity of *C. acetobutylicum*

139 towards H_2O_2 was largely dependent on Fenton chemistry (28), 1 mM of the iron chelator
140 2,2'-dipyridyl (Sigma-Aldrich) was added to attenuate peroxide-dependent killing of cells.
141 After incubation at 37°C for 30 min, the number of survived cells was determined as
142 described previously (29). Briefly, aliquots of appropriate dilutions were plated on CGM
143 medium and incubated anaerobically for 36 h at 37°C. The colony-forming units for each
144 sample were determined and normalized to the number obtained for the non-stressed wild-
145 type (100%).

146 **Mutant construction.** Gene disruption in *C. acetobutylicum* ATCC 824 was performed
147 by using group II intron-based Targetron technology as described previously (30). Briefly, a
148 350-bp fragment for retargeting an intron to insert within the *rex* gene (CAC2713) was
149 generated by one-step assembly PCR using the primers shown in Table S1 in the
150 supplemental material according to the protocol of TargeTron™ gene knockout system
151 (Sigma). The PCR product was digested and ligated to a targetron vector pWJ1 (31), yielding
152 the plasmid pWJ1-*rex*. The plasmid was methylated *in vivo* in *E. coli* ER2275 (pAN1) (32)
153 and electroporated into *C. acetobutylicum* ATCC 824. The transformants were selected on
154 CGM plate supplemented with erythromycin. The resulting mutant with an intron insertion in
155 the *rex* gene was confirmed by PCR.

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

161 **RNA isolation and real-time PCR analysis.** Total RNA was isolated from *C.*
162 *acetobutylicum* ATCC 824 grown in the P2 minimal medium with or without addition of MV
163 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). 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 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-

189 nitrilotriacetic acid affinity chromatography was performed as described previously (36). The
190 purified protein was run on a 12% sodium dodecyl sulfate-polyacrylamide gel to monitor its
191 size and purity.

192 **Electrophoretic mobility shift assay.** The 180-bp DNA fragments in the promoter
193 region of individual genes were PCR-amplified using the primers shown in Table S1. Both
194 forward and reverse primers were Cy5 fluorescence labeled at the 5´-end (Sangong Corp.,
195 Shanghai, China), and the PCR products were purified with a PCR purification kit
196 (AXYGEN). Purified Rex protein or its mutated derivative was incubated with the
197 fluorescence-labeled DNA fragment (1 nM) in 20 μl of binding buffer containing 20 mM Tris
198 (pH 7.5), 0.25 mM DTT, 10 mM $MgCl_2$, 5% glycerol, 0.8 μg bovine serum albumin (BSA),
199 and 1 μg salmon sperm DNA (non-specific competitor). Promoter fragments lacking a
200 putative Rex-binding site were used as negative controls. As potential effectors of Rex-DNA
201 binding, NADH and/or NAD^+ was added as indicated. After incubation at 30ºC for 20 min,
202 the reaction mixture was electrophoresed at 4ºC on a 6% native polyacrylamide gel in
203 0.5×Tris-borate-EDTA for 1.5 h at 100 V. Fluorescence-labeled DNA on the gel was then
204 detected with the Starion FLA-9000 (FujiFlim, Japan). For the determination of apparent
205 dissociation constants (K_d), the bands were quantified using Quantity One software, and the
206 percentage of shifted DNA was calculated. These values were plotted against the Rex
207 concentration, and K_d values were obtained using the GraphPad Prism software. All
208 determinations were performed at least in triplicate.

209 **Metabolite analysis.** For analysis of extracellular metabolites, culture samples were
210 centrifuged for 10 min at 4°C and 15,000×*g* to remove the cells. Acetone, ethanol, and
211 butanol were detected by a gas chromatograph (GC) (Agilent model 7890A) equipped with a
212 capillary column (Alltech EC-Wax; 30 m by 0.32 mm) and a flame ionization detector
213 (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 (OD_{600} of about 2.0) by centrifuging 2 ml of culture broth at 9000×*g* and 4ºC 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 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,*

239 *C. butyricum*, *C. kluyveri*, *C. novyi*, *C. perfringens*, *C. tetani*, *C. cellulovorans*, *C.*
240 *carboxidivorans*, and *C. ljungdahlii*. These 11 species with complete genome sequences
241 belong to *Clostridium* Cluster I (38), whereas they exhibit markedly different phenotypes (for
242 example, they include saccharolytic and proteolytic species as well as solventogenic and
243 acetogenic species). Rex proteins in these clostridia share close sequence homology (>68%
244 identity), and particularly the sequences of the N-terminal DNA-binding domain are highly
245 conserved (see Fig. S1 in the supplemental material). The previously identified Rex-binding
246 DNA motif in *Clostridiaceae* (24), which has consensus TTGTTAANNNNTTAACAA, was
247 used to search for Rex-binding sites in the genomes of *Clostridium* species. Finally, we
248 performed a cross-species comparison of the predicted sets of potentially co-regulated genes
249 to define the Rex regulon for each species. The candidate members and metabolic context of
250 the Rex regulons in the 11 *Clostridium* species are shown in Table 1 and Fig. 1, respectively.
251 Detailed information about the predicted DNA-binding sites, candidate Rex target genes and
252 their known or predicted transcriptional start sites is provided in Table S2 in the supplemental
253 material.

254 The reconstructed Rex regulons control the fermentation in all analyzed clostridia (Fig. 1
255 and Table 1). Most of the predicted Rex targets encode enzymes that consume NADH or
256 other reducing equivalents (e.g. reduced ferredoxin). However, the size and the specific
257 content of reconstructed Rex regulons are highly variable between different clostridial
258 species. For instance, the Rex regulon in *C. beijerinckii* constitutes 11 operons, whereas in *C.*
259 *kluyveri* Rex is predicted to control only one operon. Based on distribution of predicted Rex-
260 regulated genes in *Clostridium* species, we classified them into the conserved and variable
261 parts of Rex regulons. The conserved part of the Rex regulons includes 5 operons that are
262 potentially regulated by Rex in at least 6 species. They are *adhA* gene encoding alcohol
263 dehydrogenase, *adhE2* gene encoding bifunctional alcohol/acetaldehyde dehydrogenase, *thlA*

gene and *crt-bcd-etfBA-hbd* operon responsible for the conversion of acetyl-CoA to butyryl-CoA, and *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*, *pfor*), hydrogen production (*hydB*), TCA cycle (*frd*, *maeB*), and nitrate and sulfite reduction (*narK*, *asrABC*, *asrT*). In addition, the NAD biosynthetic genes *nadABC* were identified as candidate members of 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 set of Rex targets, including 17 genes organized in 7 operons that contain not only the known targets (*ldh*, *adhE2*, *thlA*, *crt-bcd-etfBA-hbd*) but also the newly identified members (*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 *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

289 control, the promoter fragment of *pflBA* operon in *C. acetobutylicum*, which lacks a predicted
290 Rex-binding site, was used, and no binding was observed even at 3000 nM of Rex protein
291 (Fig. 2B). The formation of Rex-DNA complex was suppressed in the presence of 400-fold
292 excess unlabeled DNA fragments but not in the presence of non-specific competitor, salmon
293 sperm DNA (data not shown). These results confirm that Rex binds specifically to the
294 promoter regions of the predicted Rex target operons in *C. acetobutylicum*.

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

303 EMSAs were also performed to assess the predicted Rex-binding sites in other analyzed
304 clostridia. For each predicted Rex target operon, the upstream candidate Rex-binding site in
305 one or two genomes was tested (Table 1). Thus 16 DNA fragments were amplified from the
306 promoter regions of *C. beijerinckii pflBA*, *adhA*, *adhA2*, *butA*, *hydB*, and *fld-pfor*, *C.*
307 *botulinum maeB*, *C. novyi ctfAB*, *C. perfringens noxE*, *C. tetani frdA*, *C. carboxidivorans*
308 *narAB*, *narK*, *codH-cooC-fhs-fchA-folD-metF-lpdA-cooC-acsDCEB*, and *grdIH*, *C.*
309 *ljungdahlii codH* and *bcd2*, respectively. These DNA fragments were tested for binding of *C.*
310 *acetobutylicum* Rex protein that is well conserved in the analyzed clostridia. A shift in the
311 presence of purified Rex was observed for all the 16 fragments (Fig. 3A and 3B). For the *C.*
312 *carboxidivorans narK* fragment, two shifted Rex-DNA complexes were detected, supporting
313 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

339 was exchanged to lysine residue by site-directed mutagenesis. The resulting Rex variant was
340 overproduced in *E. coli*, purified, and used for EMSAs. As shown in Fig. 4C, the mutated
341 protein Rex-Q51K exhibited a 12-fold increased apparent K_d value for the promoter fragment
342 of *crt-bcd-etfBA-hbd* operon. Nevertheless, when the thymine5 or adenine14 in the binding
343 sequence were substituted to guanine and cytosine, respectively, the binding affinity of the
344 mutated protein Rex-Q51K was significantly increased. Therefore, these results indicate a
345 correlation between a key amino acid residue in the DNA-binding domain of Rex proteins
346 and two nucleotides at symmetrical positions of the palindromic Rex-binding motifs. For
347 clostridial Rex proteins, the Gln51 residue in the recognition helix might position within the
348 major groove of DNA and contact with the thymine5 and adenine14 of DNA operators (see
349 Fig. S2 in the supplemental material).

350 **Effect of NADH and NAD^+ on Rex-DNA interactions**. To test if NADH and NAD^+
351 affect the interaction between the Rex from *C. acetobutylicum* and its cognate operators,
352 EMSAs were performed using the promoter fragment of *adhE2* gene. As shown in Fig. 5A,
353 the presence of only 5 μM NADH drastically decreased the formation of Rex-DNA complex,
354 whereas the addition of 1 mM NAD^+ results in a noticeable enhancement of Rex binding to
355 the DNA fragment (Fig. 5A, lanes 3 to 4). This effect is specific for NADH and NAD^+ as it
356 was not found for 10-fold higher concentrations of NADPH and $NADP^+$ (Fig. 5A, lanes 7 to
357 8). Furthermore, interaction between the same DNA fragment and *C. acetobutylicum* Rex
358 protein was assessed in the presence of physiological concentrations of NADH and NAD^+.
359 The intracellular NADH and NAD^+ pool sizes in *C. acetobutylicum* are decreased from 0.3
360 mM and 1.6 mM, respectively, during the exponential growth phase, to 0.1 mM and 1.2 mM,
361 respectively, during the subsequent solventogenic phase (39). Thus varying concentrations of
362 NADH and NAD^+, which covers the physiological concentration ranges, were used in
363 EMSAs. As shown in Fig. 5B, the DNA-binding activity of Rex was particularly susceptible

364 to changes in the NADH concentration, but NAD^+ clearly influenced the inhibitory effect of
365 NADH on Rex-DNA complex formation. These results strongly suggest that *C.*
366 *acetobutylicum* Rex senses and responds to the intracellular ratio of NADH to NAD^+ to
367 modulate its DNA-binding activities under physiological conditions.

368 **Rex negatively regulates expression of its direct target genes *in vivo*.** To validate the
369 predicted regulation of Rex on gene expression *in vivo*, the *rex* gene in *C. acetobutylicum* was
370 disrupted by insertion of an intron, resulting in the *rex*-inactivated mutant (confirmed by PCR
371 as shown in Fig. S3 in the supplemental material). The transcript levels of the predicted Rex
372 direct targets in the *rex*-inactivated mutant were compared with those in the wild-type by
373 using quantitative RT-PCR. The two strains were cultivated in minimal medium with 60 g l^{-1}
374 of glucose as carbon source, and no differences in cell growth were observed for them. For
375 comparison of transcript levels, cells were harvested in the exponential growth phase at an
376 OD_{600} of 2.0 and a growth rate of 0.16 h^{-1} for both strains, and total RNA was isolated. Six
377 qRT-PCR measurements from two independent cultures were performed. As shown in Table
378 2, the relative mRNA levels of all the 17 genes were elevated more than 1.5-fold in the *rex*-
379 inactivated mutant compared with the wild-type strain. The most prominent effect of *rex*
380 mutation was observed for the *adhE2* gene, which showed a ≥160-fold increased mRNA
381 level in the *rex*-inactivated mutant. Complementation of the *rex*-inactivated mutant by using a
382 plasmid construct constitutively expressing *rex* reduced the *adhE2* gene expression (Fig. S3).
383 The genes with a strongly increased expression in the *rex*-inactivated mutant also include the
384 *ldh* and *thlA* genes (Table 2). The *crt-bcd-etfBA-hbd*, *asrTABC*, and *nadABC* operons showed
385 a 1.5–3-fold elevated transcript level in the *rex*-inactivated mutant. Expression of the *ptb-buk*
386 operon was also increased by *rex* mutation. Therefore, the quantitative RT-PCR results
387 confirm that Rex is a negative regulator of *ldh*, *adhE2*, *thlA*, *crt-bcd-etfBA-hbd*, *ptb-buk*,

388 *nadABC*, and *asrTABC* operons involved in fermentation, NAD biosynthesis, and sulfite
389 reduction in *C. acetobutylicum*.

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

411 **Rex monitors NADH/NAD$^+$ ratio *in vivo* to regulate gene expression.** The effect of
412 exposure to MV or H_2O_2 on expression of genes in Rex regulon was compared between *C.*

413 *acetobutylicum* wild-type and *rex*-inactivated mutant strains. The transcript levels of the
414 genes involved in fermentation were determined by using quantitative RT-PCR, because the
415 fermentation genes comprise the major direct targets of clostridial Rex. As shown in Fig. 6A,
416 expression of Rex target genes *adhE2*, *thlA*, *crt*, *bcd*, and *hbd* in the wild-type was
417 significantly up-regulated by MV addition. Most strikingly, the *adhE2* gene in the wild-type
418 showed an 80-fold increased mRNA level in the presence of MV, which is consistent with
419 previous reports (6). The transcript levels of *adhE2*, *thlA*, *crt*, *bcd*, and *hbd* genes in the *rex*-
420 inactivated mutant were not significantly affected by MV addition and were higher than the
421 levels measured in MV-exposed wild-type cells (Fig. 6A). These results strongly suggest that
422 Rex responds to the increase in intracellular NADH/NAD^+ ratio achieved by MV exposure,
423 leading to derepression of Rex target genes. The *ptb*, *buk*, and *ldh* genes in the wild-type were
424 not induced when MV was present in the medium (Fig. 6A), although these genes were
425 identified as Rex direct target genes. This may be explained by possible involvement in their
426 regulation of other still unknown regulatory mechanisms in the presence of MV.

427 On the other hand, exposure to H_2O_2 resulted in 5–20-fold reduced mRNA levels of Rex
428 regulon members *adhE2*, *ldh*, *thlA*, *crt*, *bcd*, *hbd*, *ptb*, and *buk* in the wild-type (Fig. 6B).
429 These genes in the *rex*-inactivated mutant showed unaltered or 2–4-fold decreased expression
430 levels in the presence of H_2O_2 compared to the culture without H_2O_2 addition. Thus the effect
431 of H_2O_2 addition on expression of these genes in the *rex*-inactivated mutant was much
432 smaller than that in the wild-type. These results indicate that Rex represses its target genes in
433 response to the decrease in intracellular NADH/NAD^+ ratio achieved by H_2O_2 exposure.
434 Therefore, transcriptional analyses of *C. acetobutylicum* wild-type and *rex*-inactivated mutant
435 exposed to MV or H_2O_2 reveal that Rex monitors NADH/NAD^+ ratio *in vivo* to regulate
436 expression of genes in its regulon.

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

450 **Rex modulates fermentation product formation and oxidative stress tolerance in *C.***
451 ***acetobutylicum*.** To elucidate the role of Rex in regulation of central metabolism in *C.*
452 *acetobutylicum*, we compared the effect of MV exposure on fermentation product formation
453 between the wild-type and *rex*-inactivated mutant strains. As shown in Fig. 8, the *rex*-
454 inactivated mutant grew more slowly than the wild-type in the presence of MV, whereas the
455 growth rate of both strains was similar in cultures without MV addition. Determination of
456 fermentation product formation revealed different product spectra between the wild-type and
457 *rex*-inactivated mutant in the absence of MV (Fig. 8), which is in accordance with a recent
458 report (10). Mutation of *rex* resulted in a significantly increased ethanol and a slightly
459 elevated butanol production, while acetone synthesis was reduced, thus the alcohol (butanol
460 plus ethanol) to acetone ratio was improved from 2.7 to 4.8 (Fig. 8). Complementation of the
461 *rex*-inactivated mutant by using a plasmid constitutively expressing *rex* restored a typical

462 wild-type fermentation profile (Fig. S3). Alcohol formation of the wild-type was elevated by
463 22%, whereas acetone production was reduced 2.5-fold, by MV addition. By contrast,
464 exposure of the *rex*-inactivated mutant to MV resulted in only marginally increased alcohol
465 synthesis and 1.6-fold decreased acetone production (Fig. 8). Therefore, the effect of MV
466 addition on fermentation production formation in the wild-type was more profound than in
467 the *rex*-inactivated mutant. These results indicate that Rex modulates fermentation product
468 formation and plays an important role in improving the alcohol-to-acetone ratio in *C.*
469 *acetobutylicum* cultures.

470 Given that the *rex*-inactivated mutant exhibited a sharper decrease in intracellular
471 NADH/NAD^+ ratio in response to H_2O_2 exposure than the wild-type (Table 3), we wondered
472 whether Rex deficiency would influence the capability of *C. acetobutylicum* to cope with
473 oxidative stress. Hydrogen peroxide killing assays were used to assess the impact of *rex*
474 mutation on oxidative stress tolerance of *C. acetobutylicum*. The wild-type and *rex*-
475 inactivated mutant strains were incubated with an iron chelator (1 mM dipyridyl) and 50, 100,
476 or 200 μM H_2O_2 for 30 min, and the survival of cells was determined as colony forming units.
477 Results showed that the *rex*-inactivated mutant was more sensitive to H_2O_2 than the wild-type
478 (Fig. 9). The survival rate of the *rex*-inactivated mutant was approximately 2.5-fold lower
479 than that of the wild-type in the presence of 100 and 200 μM H_2O_2. Expression of a plasmid-
480 encoded *rex* from a constitutive promoter in the *rex*-inactivated mutant restored a wild-type
481 tolerance to H_2O_2. To understand why Rex deficiency increases susceptibility to oxidative
482 stress, we compared between the wild-type and *rex*-inactivated mutant strains the expression
483 levels of the genes encoding the components involved in detoxification. They include reverse
484 rubrerythrins (*rbr3A-rbr3B*), desulfoferrodoxin (*dfx*), rubredoxin (*rd*), NADH-dependent
485 rubredoxin oxidoreductase (*nror*), and the oxygen-reducing flavodiiron proteins (*fprA1* and
486 *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 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 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-

512 regulated by MV addition (Fig. 6 and 7). Consistently, previous studies have shown that the
513 *adhE2*-encoded NADH-dependent aldehyde/alcohol dehydrogenase is related to an
514 alcohologenic phenotype (41). Therefore, Rex-mediated regulation of *adhE2*, *thlA*, *crt*, *bcd*,
515 and *hbd* genes probably plays a crucial role in enhanced alcohol production in MV-exposed *C.*
516 *acetobutylicum*. In fact, we found that although MV addition resulted in a remarkable
517 increase in NADH/NAD^+ ratio in the *rex*-inactivated mutant (Table 3), it did not significantly
518 affect the expression of *adhE2*, *thlA*, *crt*, *bcd*, and *hbd* genes and its influence on
519 fermentation product formation in the *rex*-inactivated mutant was modest compared to that in
520 the wild-type (Fig. 6 and 8). The *rex*-inactivated mutant exhibited a notably reduced acetone
521 production, which is consistent with the significantly decreased transcript levels of *ctfA*, *ctfB*,
522 and *adc* genes (Fig. 7 and 8). This result suggests that Rex may modulate acetone formation
523 in *C. acetobutylicum* by regulating expression of the acetone synthesis genes, although *ctfA*,
524 *ctfB*, and *adc* genes are not Rex direct targets. Our speculation is that indirect effect of Rex
525 might occur via additional regulators such as Spo0A that is a major regulator of sporulation
526 and required for transcription of *ctfA*, *ctfB*, and *adc* genes in *C. acetobutylicum* (42, 43),
527 which is consistent with the observation of impaired spore formation for the *rex*-inactivated
528 mutant (data not shown). Therefore, our results reveal that Rex plays an important role in
529 improving the alcohol-to-acetone ratio in *C. acetobutylicum* cultures.

530 In addition to MV addition, other approaches such as carbon monoxide sparging or
531 utilization of glycerol as a substrate have also been used to shift the solvent ratio toward
532 butanol in *C. acetobutylicum* cultures. These approaches aim to inhibit hydrogenase activity,
533 and the reduction of hydrogen formation results in an increased electron flow towards butanol
534 synthesis. We speculate that Rex-dependent regulation in response to intracellular
535 NADH/NAD^+ ratio is also involved in these physiological interventions. Genetic
536 manipulations have also been applied to reduce by-product formation of *C. acetobutylicum*,

537 however, most of these attempts did not result in a desired butanol producer (44, 45). For
538 example, Jiang et al. constructed an *adc*-inactivated mutant which produced much less
539 acetone, but butanol titres were also reduced and could only be restored to the level of the
540 parent strain with pH control and MV addition to cultures (45). Based on understanding of
541 redox-dependent regulatory mechanisms, alternative engineering targets could be designed to
542 alter the intracellular redox status and improve the butanol production of *C. acetobutylicum*.

543 The strictly anaerobic clostridia can withstand limited air exposure upon activation of its
544 reductive machinery for the scavenging of ROS and molecular O_2. We found that *C.*
545 *acetobutylicum rex*-inactivated mutant is more susceptible to H_2O_2 killing than the wild-type
546 (Fig. 9), indicating that Rex modulates oxidative stress tolerance in this obligate anaerobe.
547 Although involvement of Rex in regulation of oxidative stress response has also been
548 reported for facultative anaerobe *S. mutans* (15), the mechanism may be different between *S.*
549 *mutans* and clostridia. Our results demonstrate that Rex responds to the decrease in
550 intracellular NADH/NAD^+ ratio achieved by H_2O_2 exposure to repress its target genes
551 including those encoding NADH-consuming enzymes in central metabolism (e.g. *ldh*, *adhE2*,
552 *bcd-etfBA*, and *hbd*) (Fig. 6). This may increase availability of reducing power needed for
553 reduction of H_2O_2. Moreover, expression of the genes encoding the components involved in
554 detoxification of ROS and oxygen was down-regulated in the *rex*-inactivated mutant (Fig. 10),
555 although these genes are not preceded by Rex-binding sites, suggesting that Rex could
556 indirectly enhance the detoxification system in *C. acetobutylicum*. Because these genes are
557 primary targets of transcriptional repressor PerR in *C. acetobutylicum* according to a previous
558 study (46), we speculate that Rex may regulate expression of these genes via PerR or other
559 transcription factors. However, more work is needed to elucidate the mechanism of the
560 involvement of Rex in regulation of oxidative stress response. The Rex and PerR regulatory
561 systems that both are widely distributed in *Clostridium* species seem to play important roles

562 in the oxidative stress defence in *C. acetobutylicum*, but they sense different signals and
563 possess different direct targets. Whereas Rex senses intracellular NADH/NAD^+ ratio to
564 regulate many fermentation genes, PerR is a peroxide sensor that negatively controls
565 expression of the genes involved in the oxygen and ROS detoxification.

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

585 This study gains an insight into the potential regulatory role of Rex in clostridial species
586 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 coordinately 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

612 solventogenic clostridia may be partly attributed to the variability of the Rex regulon
613 members.

614 The acetogenic *C. carboxidivorans*, *C. ljungdahlii*, and *Clostridium autoethanogenum* are
615 capable of using the Wood-Ljungdahl pathway to fix CO_2 or CO and convert it into acetyl-
616 CoA. This feature makes them become promising production strains for industrial syngas
617 fermentations (49). However, regulation of the Wood-Ljungdahl pathway genes in these
618 acetogenic clostridia remains to be explored. Here we predicted a candidate Rex-binding site
619 located upstream of the Wood-Ljungdahl pathway gene cluster in the genomes of *C.*
620 *carboxidivorans* and *C. ljungdahlii*. Binding of Rex to the promoter region of this gene
621 cluster in both clostridia was verified by EMSAs (Fig. 3). A putative Rex-binding site
622 upstream of the Wood-Ljungdahl pathway gene cluster was also identified in the genome of
623 *C. autoethanogenum* (data not shown). This suggests that Rex may play a role in regulation
624 of CO or CO_2 reduction in *C. carboxidivorans*, *C. ljungdahlii*, and *C. autoethanogenum*.
625 Whether *rex* inactivation will lead to derepression of Wood-Ljungdahl pathway genes and
626 improvement of syngas fermentation in these acetogenic clostridia needs to be tested.
627 Nevertheless, this study offers an insight into redox-dependent gene regulation in these
628 species, which could be useful for designing sophisticated metabolic engineering approaches
629 to increase the product yields of syngas fermentation.
630

631 ## ACKNOWLEDGEMENTS

632 We thank Qing Zhang from University of Arkansas for Medical Sciences for helpful
633 discussions. This work was supported in part by the National Basic Research Program of
634 China (973: 2012CB721101) and the National Natural Science Foundation of China
635 (31121001 and 31470168).
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798
799
800

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

Operon and metabolism	*Clostridium* species											Functional role
	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	
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-pfor	−	**+***	−	+	−	−	−	−	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
Others												
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

802 [a] The genes preceded by a conserved Rex-binding site are indicated by +, and the predicted Rex-
803 binding sites verified by targeted experiments are marked by bold type and asterisk. Genes without a
804 candidate Rex binding site are indicated by −. The absence of orthologous gene(s) in the analyzed
805 genomes is indicated by 0.
806

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

Gene name	Gene ID	mRNA ratio[a] (*rex* mutant /WT)
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

809 [a] Data represent means ± S.D. of values of mRNA ratios obtained from six measurements starting
810 from two independent cultures. The strains were cultivated in the P2 minimal medium, and total RNA
811 was isolated in the exponential growth phase at an OD_{600} of about 2.0. The *p* value of the mRNA
812 ratios for all the genes studied is smaller than 0.01.
813

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

Strains	Addition to cultures[a]	NADH (mM)	NAD^+ (mM)	NADH/NAD^+
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

816 [a] Data represent means ± S.D. values of nine measurements from three biological replicates. The
817 strains were grown in the P2 minimal medium without or with addition of 1 mM MV or 30 μM H_2O_2.
818 The intracellular concentrations of NADH and NAD^+ were determined in the exponential growth
819 phase at an OD_{600} of about 2.0.
820

821 **FIGURE LEGENDS**

822

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

828

829 **FIG 2** EMSAs with purified Rex protein and DNA fragments from the promoter regions of
830 predicted target genes in *C. acetobutylicum*. (A) DNA fragments (1 nM) from the promoter
831 regions of *C. acetobutylicum adhE2*, *ldh*, *crt*, *thlA*, *asrT*, *ptb*, and *nadA* genes were
832 fluorescence-labeled and incubated with the indicated concentrations of Rex protein for 20
833 min at 30ºC. Then the protein-DNA complexes were resolved by electrophoresis on native
834 6% polyacrylamide gels. Quantification of the bands allowed the determination of the
835 apparent K_d values (see MATERIALS AND METHODS). The values shown represent the
836 average and standard deviation of at least three independent assays. (B) As a negative control
837 the promoter region of *C. acetobutylicum pflBA* operon, which lacks a putative Rex binding
838 site, was used.

839

840 **FIG 3** EMSAs with purified Rex protein and the promoter regions of predicted target genes
841 in *Clostridium* species other than *C. acetobutylicum*. (A) EMSAs were performed in the
842 absence (lane 1) and in the presence of 60, 300, and 1000 nM of Rex protein (lanes 2 to 4).
843 (B) EMSAs were performed in the absence (lane 1) and in the presence of 500, 1000, and
844 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_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 the P2 minimal medium without or with addition of 1 mM MV or 30 μM

870 H_2O_2. Total RNA was isolated from cells harvested in the exponential growth phase at an
871 OD_{600} of about 2.0. The mRNA levels of each gene were determined by qRT-PCR and
872 normalized to the gene expression in the wild-type strain grown in the absence of MV or
873 H_2O_2. Data represent means ± S.D. of values from six measurements starting from two
874 independent cultures. Differences in the mRNA levels of *adhE2*, *thlA*, *crt*, *bcd*, and *hbd* genes
875 in the wild-type between the absence and presence of MV are statistically significant ($p <$
876 0.01), while the mRNA levels of all the studied genes in the wild-type are significantly
877 different ($p < 0.01$) upon exposure to H_2O_2.
878
879 **FIG 7** Effect of *rex* inactivation and MV addition on transcript levels of the fermentation
880 genes that are not members of the predicted Rex regulon. Data represent means ± S.D. of
881 values from six measurements starting from two independent cultures and are normalized to
882 the expression level in the wild-type without MV exposure. Differences in the mRNA levels
883 of *adhE1*, *ctfA*, *ctfB*, and *adc* genes between the wild-type and *rex*-inactivated mutant are
884 statistically significant ($p < 0.01$), while the mRNA levels of all the studied genes in the wild-
885 type are significantly different ($p < 0.01$) upon treatment with MV.
886
887 **FIG 8** Cell growth and fermentation product formation in batch cultures of *C. acetobutylicum*
888 wild-type and *rex*-inactivated mutant strains without or with addition of MV. The strains
889 were grown in the P2 minimal medium containing 60 g l^{-1} of glucose. At an OD_{600} of about
890 0.15, MV was added to a final concentration of 1 mM. Cell growth was monitored
891 spectrophotometrically at 600 nm. Formation of acetone, butanol, and ethanol was
892 determined by gas chromatography. The data points and error bars represent means ± S.D. of
893 values from three independent cultures.
894

895 **FIG 9** Survival of *C. acetobutylicum* wild-type, *rex*-inactivated mutant, and *rex*-
896 complemented strains after hydrogen peroxide treatment. The strains were grown in P2
897 minimal medium to an OD_{600} of about 2.0. Then cells were exposed to 1 mM of the iron
898 chelator 2,2'-dipyridyl and the indicated concentrations of H_2O_2 or the equal volume of H_2O.
899 After incubation at 37°C for 30 min, the colony-forming units were determined as the
900 survival of cells and normalized to the number obtained for the non-stressed wild-type. Data
901 represent means ± S.D. of values from three independent experiments.
902
903 **FIG 10** Influence of *rex* inactivation on transcript levels of the genes involved in
904 detoxification of ROS and molecular O_2 in *C. acetobutylicum*. Total RNA was isolated from
905 the wild-type, *rex*-inactivated mutant, and *rex*-complemented strains grown in the P2 minimal
906 medium and harvested at an OD_{600} of about 2.0. The expression levels of each gene were
907 normalized to the gene expression in the wild-type strain. Data represent means ± S.D. of
908 values from six measurements starting from two independent cultures. Differences in the
909 mRNA levels of all the studied genes between the wild-type and *rex*-inactivated mutant are
910 statistically significant ($p < 0.01$).

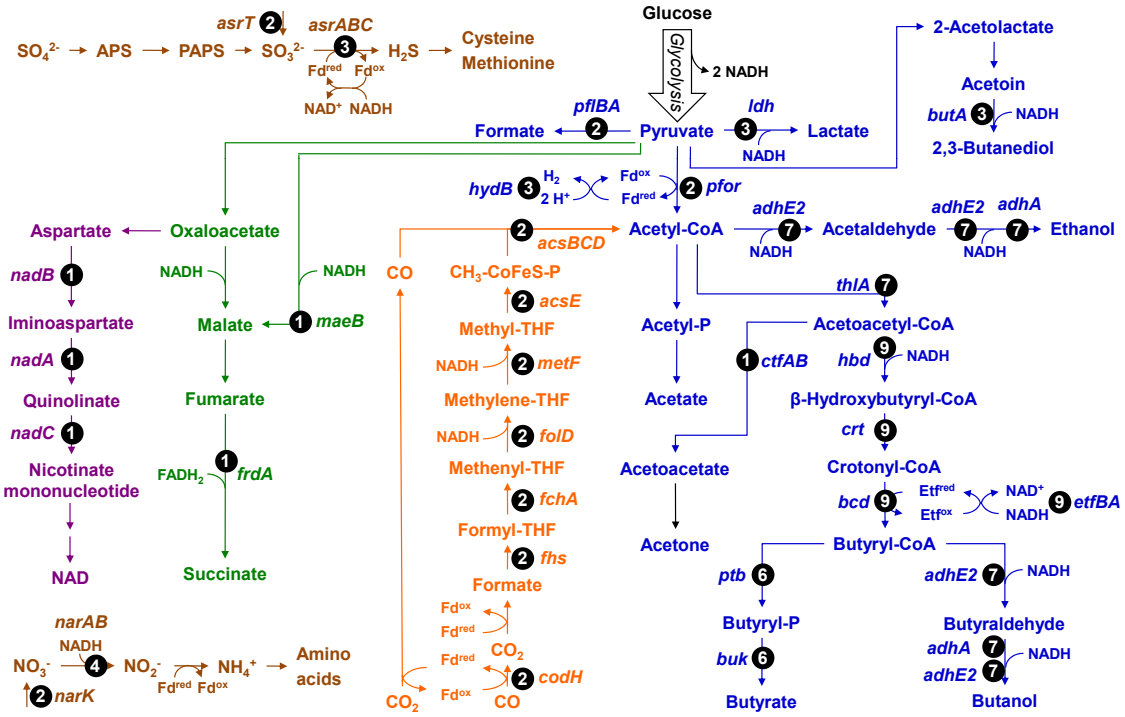

Fig. 1.

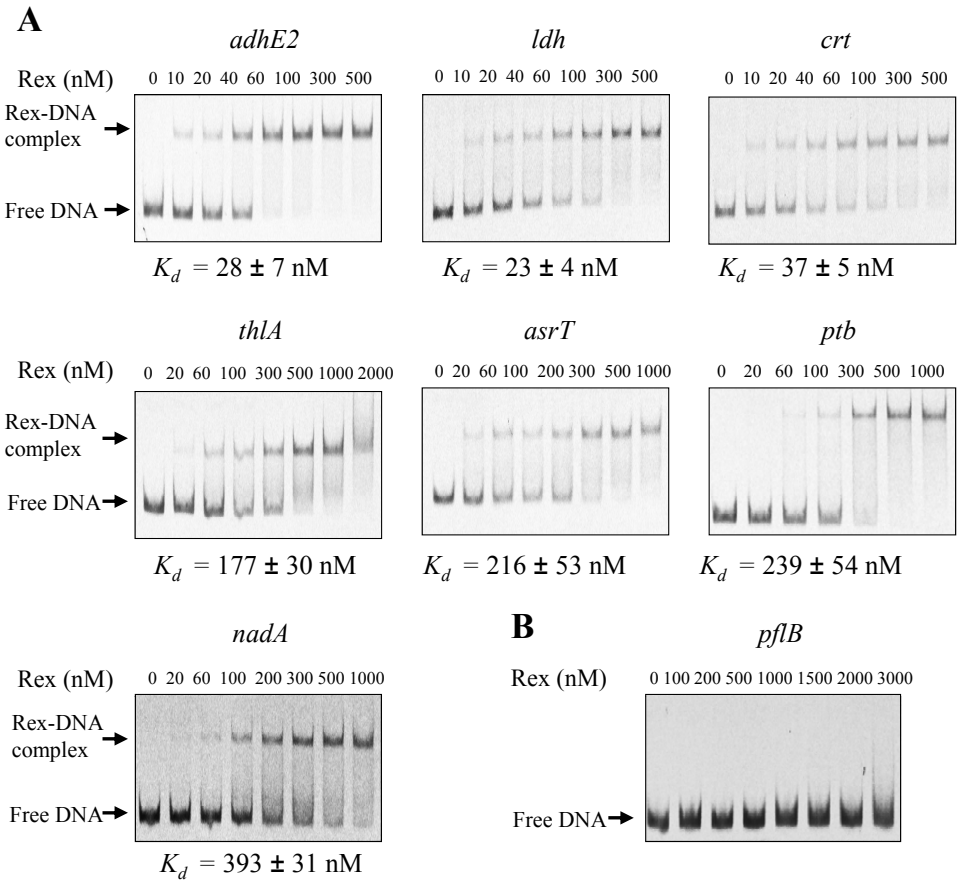

Fig. 2.

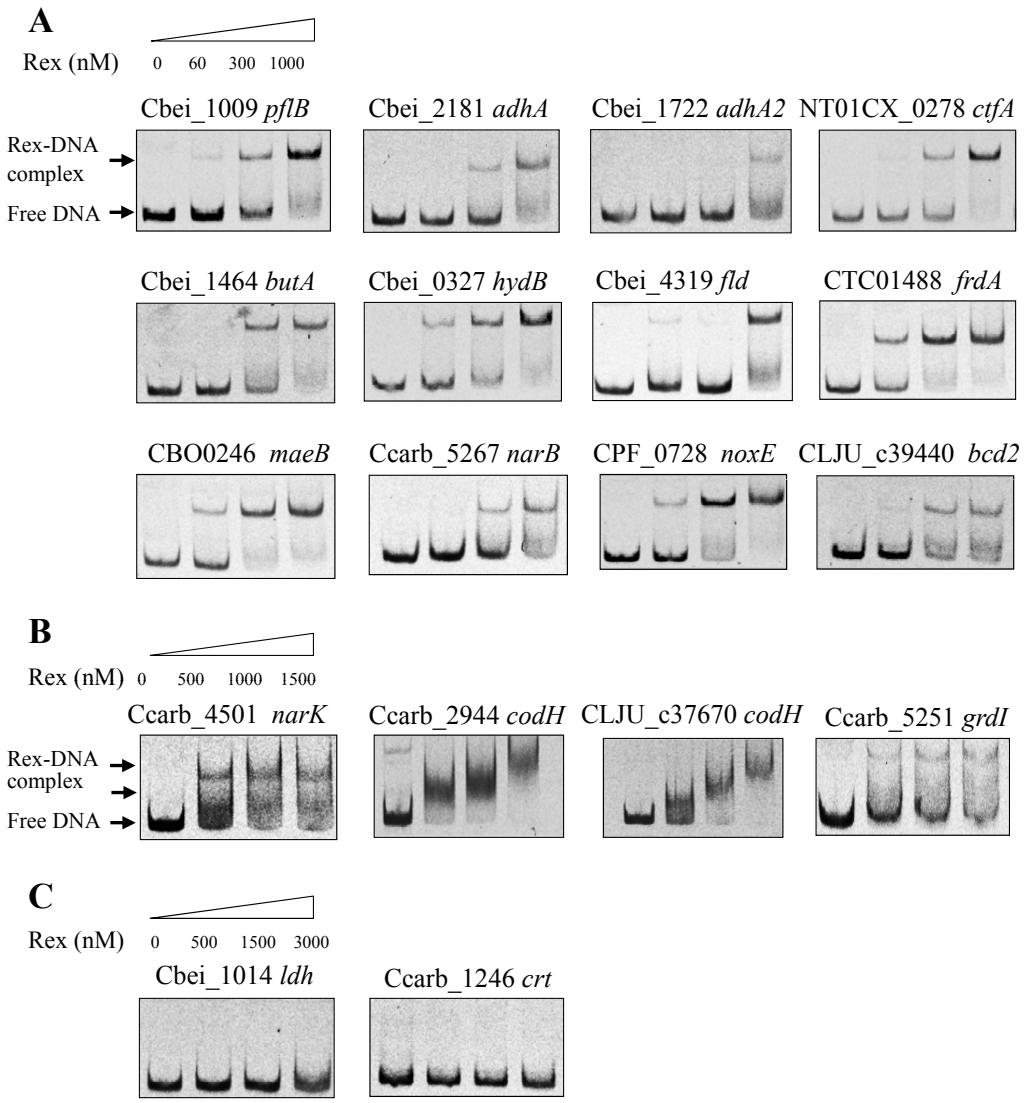

Fig. 3.

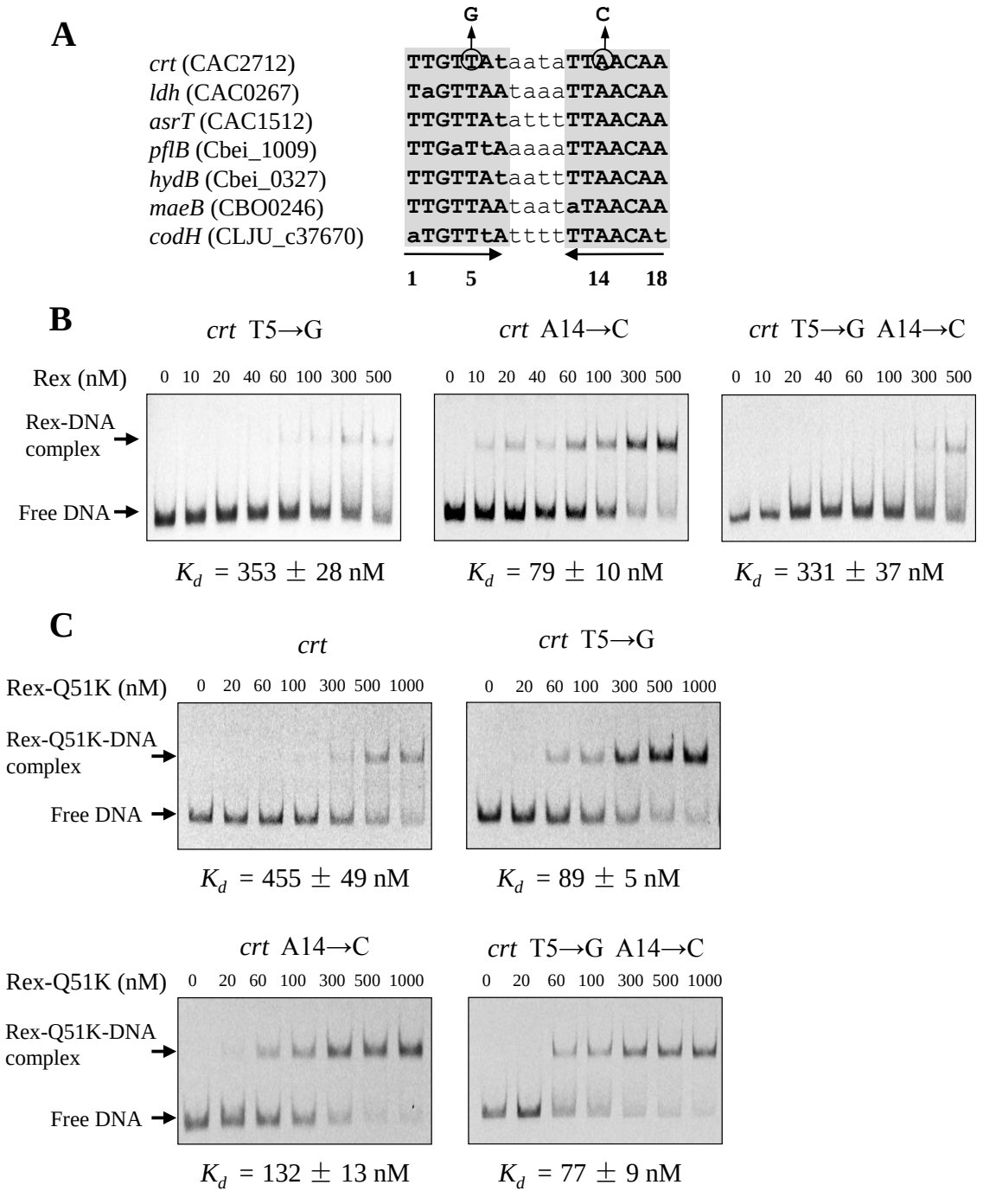

Fig. 4.

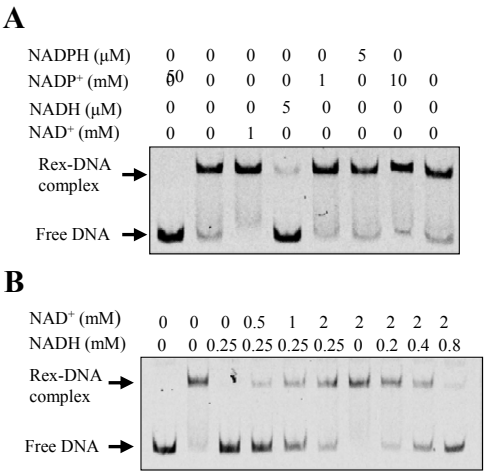

Fig. 5.

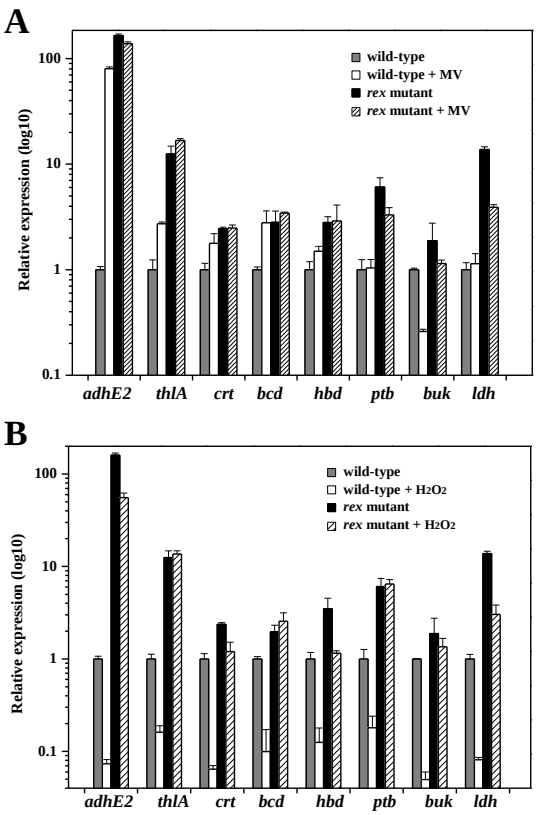

Fig. 6.

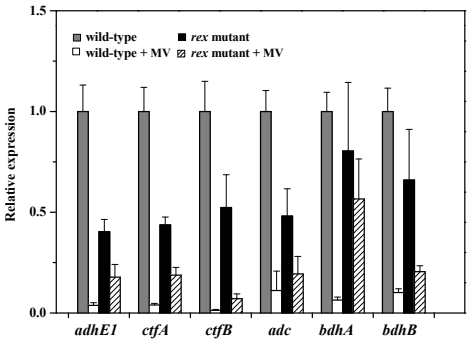

Fig. 7.

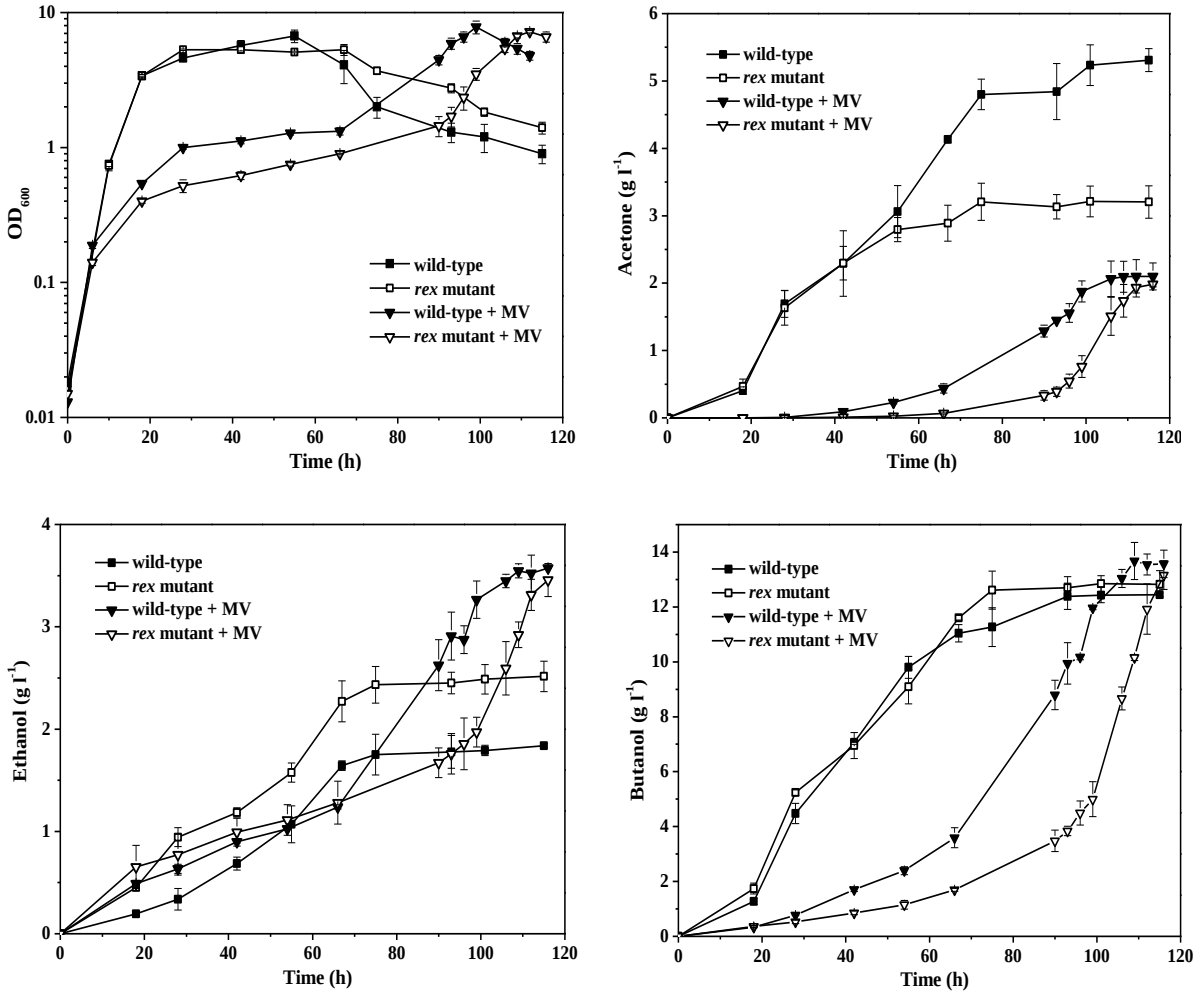

Fig. 8.

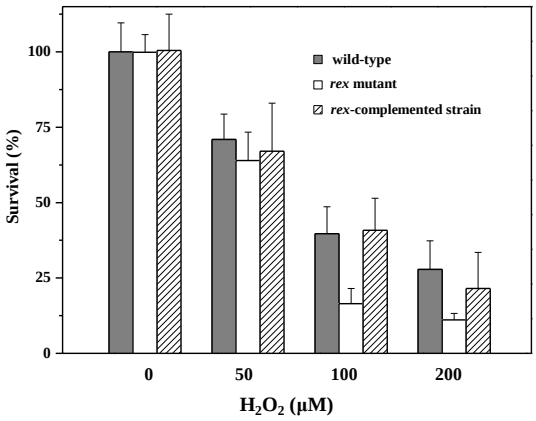

Fig. 9.

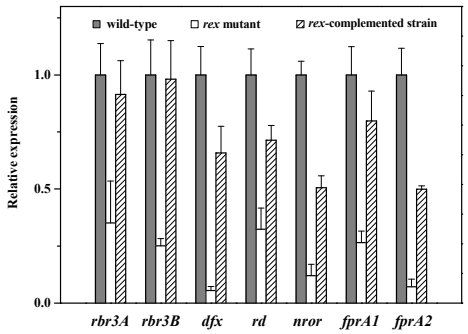

Fig. 10.