# A Novel Dual-*cre* Motif Enables Two-Way Autoregulation of CcpA in *Clostridium acetobutylicum*

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**ABSTRACT** The master regulator CcpA (catabolite control protein A) manages a large and complex regulatory network that is essential for cellular physiology and metabolism in Gram-positive bacteria. Although CcpA can affect the expression of target genes by binding to a *cis*-acting catabolite-responsive element (*cre*), whether and how the expression of CcpA is regulated remain poorly explored. Here, we report a novel dual-*cre* motif that is employed by the CcpA in *Clostridium acetobutylicum*, a typical solventogenic *Clostridium* species, for autoregulation. Two *cre* sites are involved in CcpA autoregulation, and they reside in the promoter and coding regions of CcpA. In this dual-*cre* motif, $cre_P$, in the promoter region, positively regulates *ccpA* transcription, whereas $cre_{ORF}$, in the coding region, negatively regulates this transcription, thus enabling two-way autoregulation of CcpA. Although CcpA bound $cre_P$ more strongly than $cre_{ORF}$ *in vitro*, the *in vivo* assay showed that $cre_{ORF}$-based repression dominates CcpA autoregulation during the entire fermentation. Finally, a synonymous mutation of $cre_{ORF}$ was made within the coding region, achieving an increased intracellular CcpA expression and improved cellular performance. This study provides new insights into the regulatory role of CcpA in *C. acetobutylicum* and, moreover, contributes a new engineering strategy for this industrial strain.

**IMPORTANCE** CcpA is known to be a key transcription factor in Gram-positive bacteria. However, it is still unclear whether and how the intracellular CcpA level is regulated, which may be essential for maintaining normal cell physiology and metabolism. We discovered here that CcpA employs a dual-*cre* motif to autoregulate, enabling dynamic control of its own expression level during the entire fermentation process. This finding answers the questions above and fills a void in our understanding of the regulatory network of CcpA. Interference in CcpA autoregulation leads to improved cellular performance, providing a new useful strategy in genetic engineering of *C. acetobutylicum*. Since CcpA is widespread in Gram-positive bacteria, including pathogens, this dual-*cre*-based CcpA autoregulation would be valuable for increasing our understanding of CcpA-based global regulation in bacteria.

**KEYWORDS** CcpA, *Clostridium acetobutylicum*, dual-*cre* motif, two-way autoregulation

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Transcriptional autoregulation is an essential feature for some key regulatory proteins in bacteria, enabling precise feedback control of their expressional levels in response to extra- or intracellular signals (1–3). Autoregulation can be positive or negative, and both play important roles in genetic networks. For example, in the most-studied organism, *Escherichia coli*, a variety of transcription factors (TFs) that are involved in substrate utilization (2, 3), DNA replication (1), polysaccharide capsule

formation (4), and two-component systems (5, 6) are capable of dynamically controlling their own transcription. Autoregulated TFs have also been discovered in several other microorganisms, including pathogens (7–9). However, the vast majority of these auto-regulated TFs are pathway specific and rarely act globally.

Catabolite control protein A (CcpA) is an essential global regulator involved in mediating many important cellular processes in Gram-positive bacteria, including pathogens (10–12). CcpA targets genes by recognizing a 14- to 16-nucleotide (nt) *cre* (catabolite-responsive element) site in the coding or promoter regions (13). In previous work, we identified the CcpA protein in *Clostridium acetobutylicum*, a typical solvento-genic *Clostridium* species that has been widely used in industrial *a*cetone-*b*utanol-*e*thanol (ABE) production (14). During the following analysis, many potential CcpA-binding *cre* sites were uncovered, and a *cre* was found inside the coding region for CcpA (15). We recently scanned the surrounding regions of CcpA more carefully, and in addition to the above-mentioned *cre* site inside the CcpA-coding region, a noncanoni-cal CcpA-binding site may be present in the promoter region. If this is true, this new CcpA-binding site, combined with the *cre* inside the CcpA-coding region, will yield a novel dual-*cre* motif that enables the autoregulation of CcpA in *C. acetobutylicum*. In *Bacillus subtilis*, CcpA seems to be constitutively expressed (16); however, it is still unclear whether and how CcpA expression is regulated in other Gram-positive bacteria, although in *Lactobacillus pentosus* and *Staphylococcus xylosus*, potential single *cre* sites have been detected in the *ccpA* promoter region (17, 18). Understanding the dual-*cre* form employed by the *C. acetobutylicum* CcpA may assist in answering these regulation questions.

In this work, we performed a detailed investigation into how CcpA employs a novel dual-*cre* motif to achieve its autoregulation in *C. acetobutylicum*. The results from *in vitro* and *in vivo* experiments showed that the *cre* sites in the promoter and the coding regions activate and repress, respectively, CcpA expression. These two *cre* sites bind CcpA with different affinities, suggesting a potential leveraging of these two sites in CcpA autoregulation. Based on the understanding of dual-*cre*-based CcpA autoregula-tion in *C. acetobutylicum*, we modulated this autoregulation process by mutating the *cre* site in the coding region, releasing the self-repression effect on CcpA expression, and this novel engineering strategy led to an encouraging improved performance of *C. acetobutylicum* in growth and product synthesis.

## RESULTS

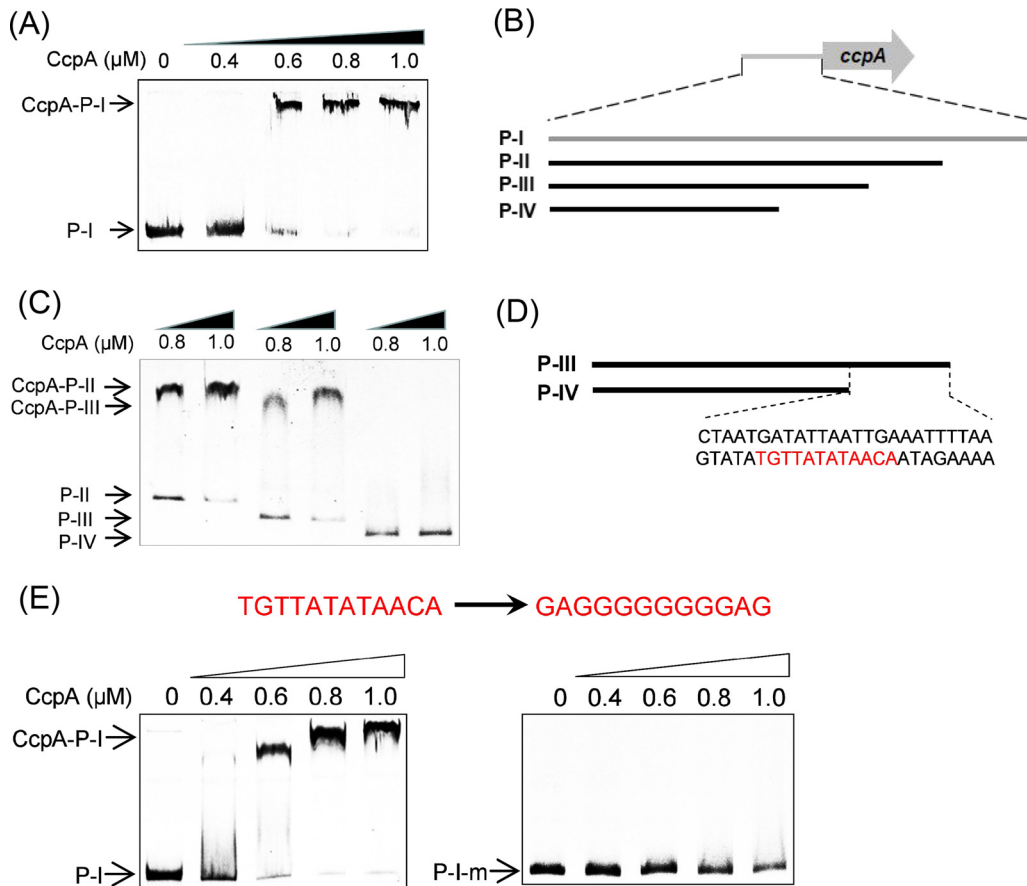**Identification of the *cre* site inside the CcpA-coding region in *C. acetobutylicum*.** In our previous work, the pleiotropic regulatory functions of CcpA were uncovered in *C. acetobutylicum*, yielding a large number of genes that CcpA targets as well as a consensus motif of CcpA-binding *cre* sites (15). A possible *cre* sequence (AAGAAAAAG ATTACAT) was found in the CcpA-coding region, but confirmation of its function awaits experimental verification. We name this putative *cre* site "*cre*$_{ORF}$." If *cre*$_{ORF}$ is really a CcpA-binding site, CcpA in *C. acetobutylicum* can regulate itself at the transcriptional level. Such an autoregulation mechanism, to our knowledge, has not been reported in CcpA, the global regulator in Gram-positive bacteria.

To verify this hypothesis, we first examined the binding of CcpA to *cre*$_{ORF}$ by using electrophoretic mobility shift assays (EMSAs). The whole sequence of the CcpA-coding region (1,005 bp) was divided into three fragments that overlapped each other by 50 bp (Fig. 1A); these fragments were used as probes in the EMSAs. As expected, a substantial DNA binding shift was observed in the case in which the first 400-bp segment (O-I) (which contains just the *cre*$_{ORF}$ sequence) was used (Fig. 1B), whereas no obvious DNA binding shift was detected for the other DNA probes, i.e., O-II and O-III (Fig. 1B). When *cre*$_{ORF}$ was mutated, this 400-bp DNA fragment was not bound by CcpA (Fig. 1C), further demonstrating the binding between CcpA and *cre*$_{ORF}$. Together, these results verified that *cre*$_{ORF}$ is a major binding site of CcpA, although other binding sites with very low affinity to CcpA in its coding region may exist.
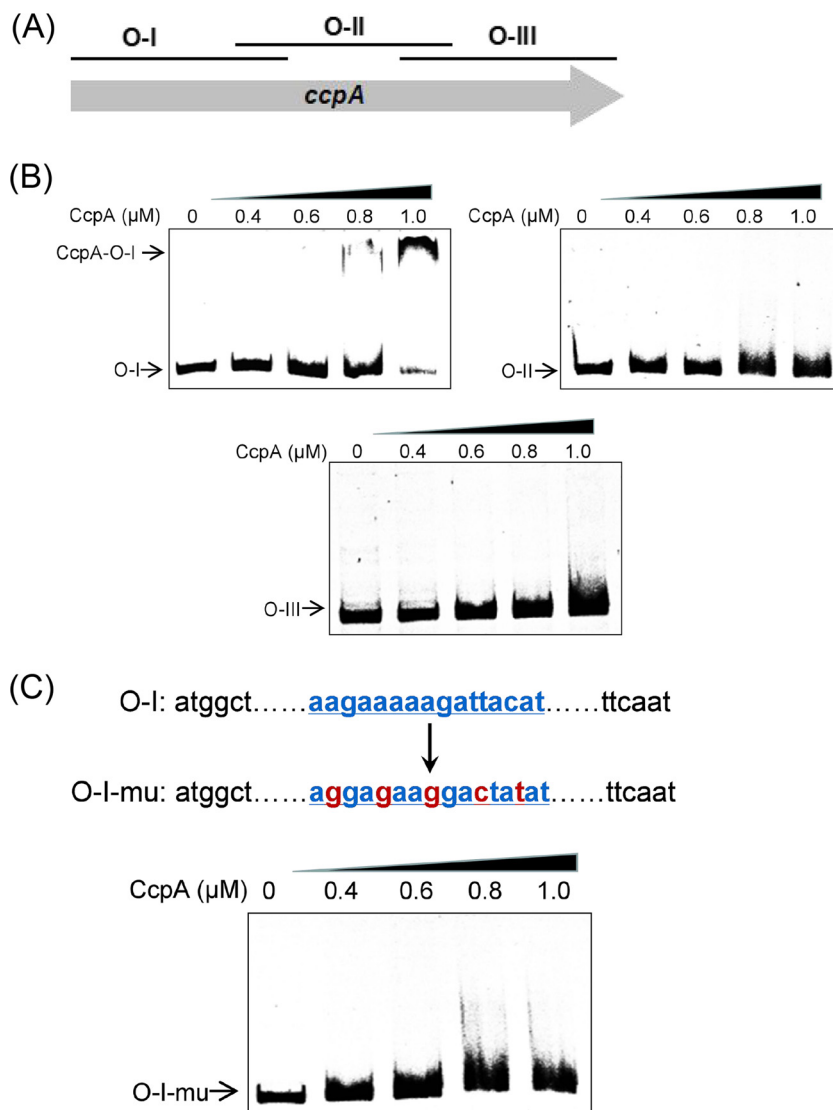
**FIG 1** Identification of the CcpA-binding site inside the *ccpA* coding region. (A) The sequence of the whole *ccpA* coding region was divided into three fragments, O-I (400 bp), O-II (400 bp), and O-III (305 bp). (B) EMSAs for investigating the *in vitro* CcpA binding to O-I, O-II, and O-III. (C) Mutation of the potential CcpA-binding site (AAGAAAAAGATTACAT) inside the O-I fragment and EMSAs for investigating the *in vitro* CcpA binding to fragment O-I-mu, which contained a mutated *cre*$_{ORF}$ site. The final concentration of Cy5-labeled DNA probe used was 0.04 pM, and 0 to 1.0 $\mu$M CcpA was used.

**Identification of a noncanonical *cre* site in the CcpA promoter region.** Since *cre*$_{ORF}$ is the *cre* site in the CcpA-coding region, we sought potential *cre* sites in the promoter region of *ccpA* despite negative results from previous genome screening based on the typical *cre* motif (15). EMSAs using the whole promoter region (P-I) sequence as a probe were performed to find these sites. An obvious DNA binding shift was observed (Fig. 2A), indicating the existence of potential noncanonical CcpA-binding sites in the promoter region. To localize these noncanonical *cre* sites, the 244-bp promoter region of *ccpA* was gradually truncated, yielding the fragments P-I (244 bp), P-II (194 bp), P-III (154 bp), and P-IV (104 bp) as probes for EMSAs (Fig. 2B). Figure 2C shows that a significantly shifted band was observed in assays with the P-II and P-III probes, while the band was almost completely eliminated when the P-IV probe was used, thereby indicating that a potential CcpA-binding site resides in the truncated fragment from the P-III region. Since this truncated fragment is only 50 bp long, we visually scanned this region and found a 12-bp palindromic sequence (TGTTATATAA

**FIG 2** Identification of the noncanonical CcpA-binding site inside the promoter region of *ccpA*. (A) EMSAs for investigating the *in vitro* CcpA binding to the promoter region (P-I). (B) Truncation of P-I. Fragments of 50, 100 and 150 bp in length were cut off from P-I, yielding the three shortened fragments P-II, P-III, and P-IV. (C) EMSAs for investigating the *in vitro* CcpA-binding to P-II, P-III, and P-IV. (D) Detection of a potential CcpA-binding site in the truncated fragment from P-III via visual analysis. The putative binding site (TGTTATATAACA) is highlighted in red. (E) EMSAs for investigating the *in vitro* CcpA binding to the probe P-I and its derivative P-I-m, in which TGTTATATAACA was mutated to GAGGGGGGGGAG. The final concentration of Cy5-labeled DNA probe used was 0.04 pM, and 0 to 1.0 $\mu$M CcpA was used.

CA), which we named "$cre_P$" (Fig. 2D). The $cre_P$ site is quite different from the typical *cre* site as well as the newly identified, flexible $cre_{var}$ architecture in *C. acetobutylicum* (15, 19). To further examine whether $cre_P$ is a CcpA-binding site, it was mutated, which resulted in a derived P-I fragment (P-I-mu) that was used as the probe in EMSAs (Fig. 2E). As expected, the mutation of $cre_P$ almost completely abolished the band shift of CcpA-P-I (Fig. 2E), thereby verifying the role of $cre_P$.

Given that $cre_P$ is a noncanonical CcpA-binding site, we next sought to determine the contribution of each nucleotide in $cre_P$ to CcpA binding. Each nucleotide in $cre_P$ was mutated individually, and the resulting 12 derivative probes were used in EMSAs (Fig. 3A). The EMSA results showed that compared to the binding between CcpA and the original probe, P-I (Fig. 2E), the CcpA-P-I binding of all $cre_P$ mutants was weakened, and while the magnitude of weakening differed among mutation sites, it was particularly obvious for the two outermost nucleotides (M-L1 and M-R6) (Fig. 3B). Simultaneous mutations at both L1 and R6 were also performed (Fig. 3A), and the resulting probe (M-L1-R6) was used to examine its binding to CcpA. As expected, this double-nucleotide mutation completely eliminated the CcpA-$cre_P$ binding (Fig. 3B), further indicating the importance of the nucleotides at L1 and R6 site for $cre_P$. Therefore, it can be concluded that each nucleotide within $cre_P$ contributes to CcpA-$cre_P$ binding; moreover, this binding is influenced more by the outer nucleotides than by the inner nucleotides.

## (A)

| | L1 | L2 | L3 | L4 | L5 | L6 | R1 | R2 | R3 | R4 | R5 | R6 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *cre*P | T | G | T | T | A | T | A | T | A | A | C | A |
| M-L1 | G | G | T | T | A | T | A | T | A | A | C | A |
| M-L2 | T | A | T | T | A | T | A | T | A | A | C | A |
| M-L3 | T | G | G | T | A | T | A | T | A | A | C | A |
| M-L4 | T | G | T | G | A | T | A | T | A | A | C | A |
| M-L5 | T | G | T | T | G | T | A | T | A | A | C | A |
| M-L6 | T | G | T | T | A | G | A | T | A | A | C | A |
| M-R1 | T | G | T | T | A | T | G | T | A | A | C | A |
| M-R2 | T | G | T | T | A | T | A | G | A | A | C | A |
| M-R3 | T | G | T | T | A | T | A | T | G | A | C | A |
| M-R4 | T | G | T | T | A | T | A | T | A | G | C | A |
| M-R5 | T | G | T | T | A | T | A | T | A | A | A | A |
| M-R6 | T | G | T | T | A | T | A | T | A | A | C | G |
| M-L1-R6 | G | G | T | T | A | T | A | T | A | A | C | G |

## (B)
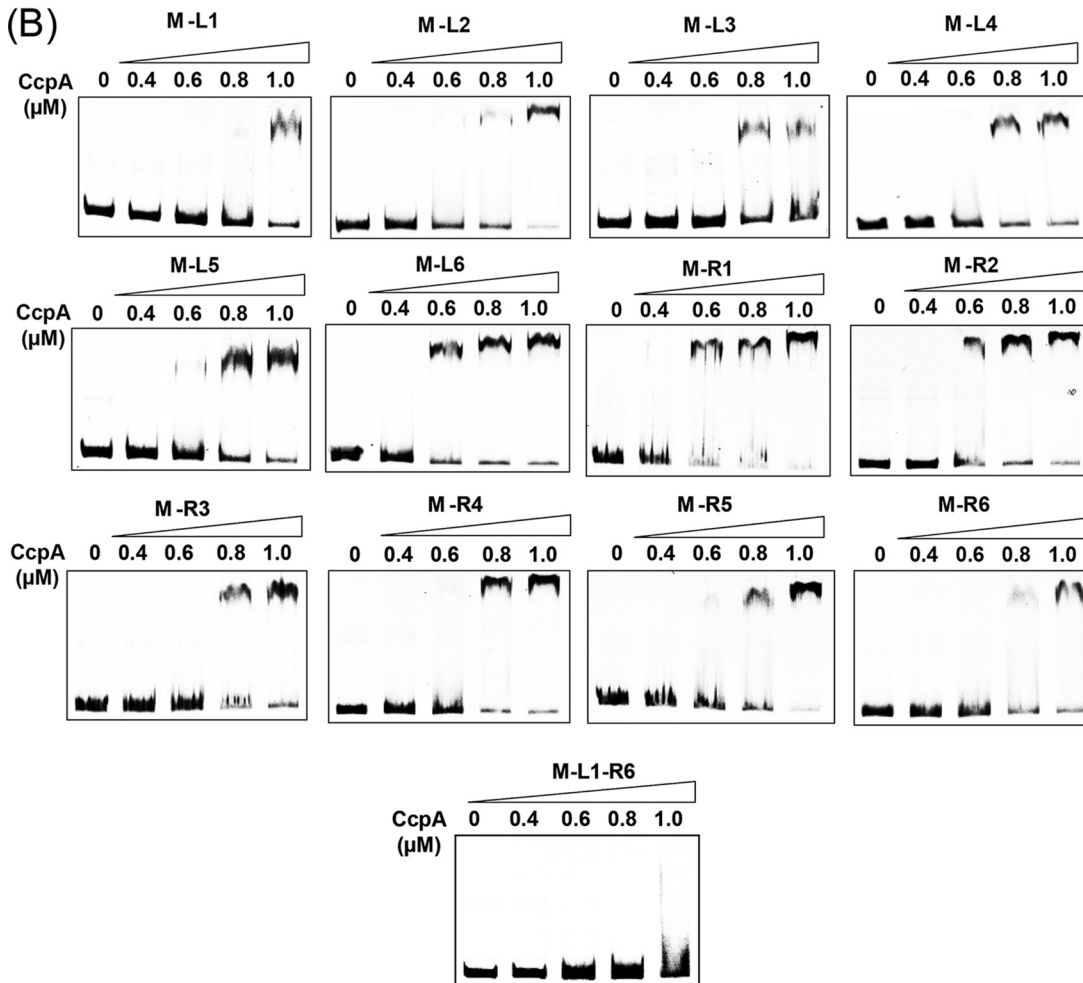


**FIG 3** Characterization of the noncanonical CcpA-binding site *cre*P. (A) Single and double point mutations of *cre*P. The mutation sites are marked in red. (B) EMSAs of CcpA binding to the probes containing the mutated *cre*P sites. The final concentration of Cy5-labeled DNA probe used was 0.04 pM, and 0 to 1.0 $\mu$M CcpA was used.

**Characterization of dual-*cre*-based CcpA autoregulation.** These results strongly suggested that CcpA employs a novel dual-*cre* (*cre*P and *cre*ORF) motif to regulate CcpA expression (Fig. 4A). This autoregulation model was then investigated *in vivo*. The promoter fragment and the coding region of CcpA, containing mutated *cre*P and *cre*ORF
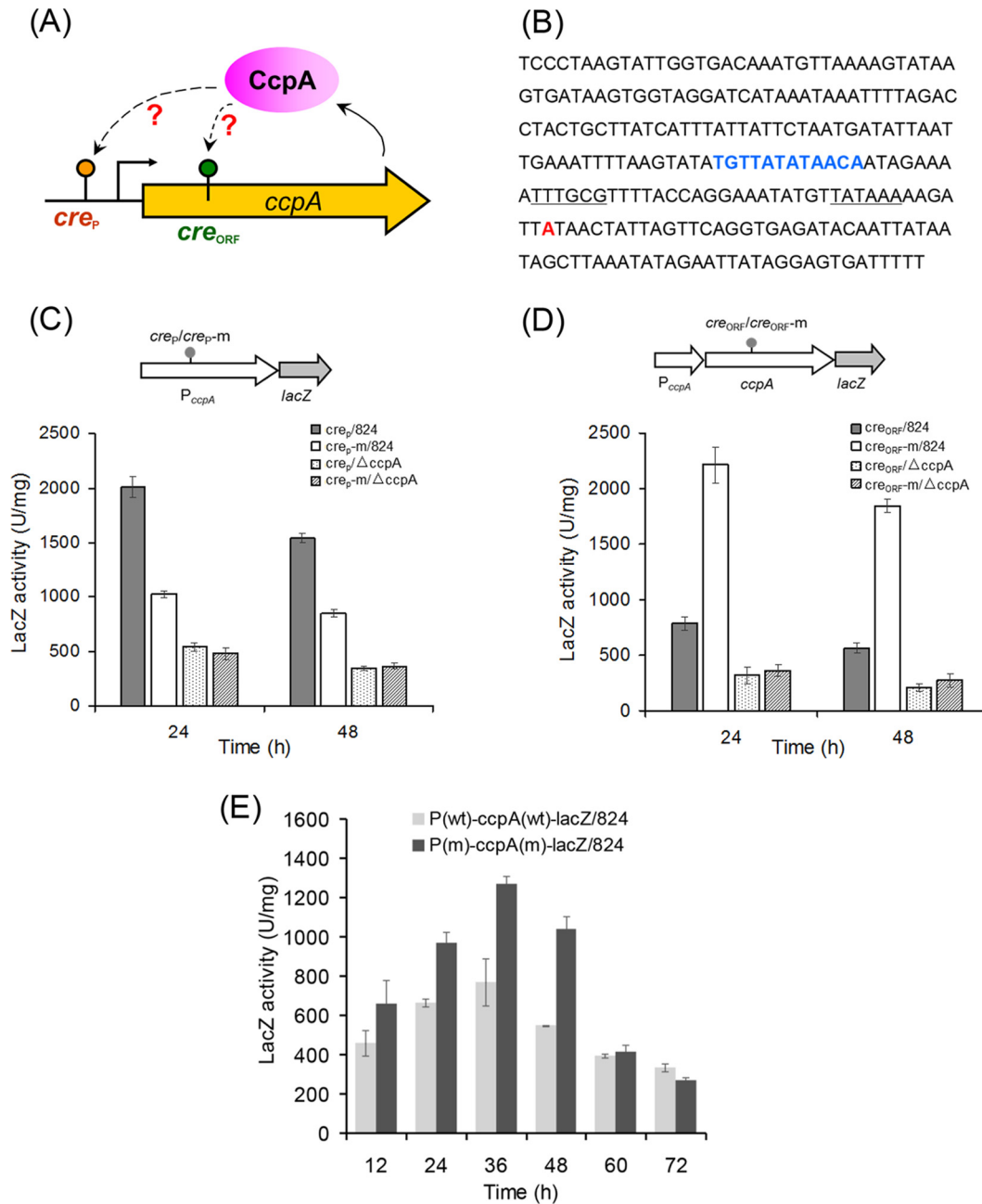
**FIG 4** Characterization of the roles of the dual *cre* sites in CcpA autoregulation. (A) The proposed dual-*cre* form for CcpA autoregulation. (B) Characteristics of the promoter region of *ccpA*. The transcriptional start site A is highlighted in red. The −10 and −35 regions are underlined. The CcpA-binding site *cre*<sub>P</sub> is highlighted in blue. (C) Role of the *cre*<sub>P</sub> site in controlling CcpA expression. *cre*<sub>P</sub>, original promoter sequence of *ccpA*; *cre*<sub>P</sub>-m, derived promoter sequence of *ccpA* that was mutated in the *cre*<sub>P</sub> site; 824, *C. acetobutylicum* wild-type strain; ΔccpA, *C. acetobutylicum* strain with *ccpA* inactivation. *lacZ* was used here as the reporter gene, and the gray circle represents the original and mutated CcpA-binding sites, *cre*<sub>P</sub> and *cre*<sub>P</sub>-m. P<sub>*ccpA*</sub>, promoter of the *ccpA* gene. (D) Role of the *cre*<sub>ORF</sub> site in controlling CcpA expression. *cre*<sub>ORF</sub>, *ccpA* coding region; *cre*<sub>ORF</sub>-m, derived *ccpA* coding region that was mutated in the *cre*<sub>ORF</sub> site. The gray circle represents the original and mutated CcpA-binding sites, *cre*<sub>ORF</sub> and *cre*<sub>ORF</sub>-m. The *ccpA* and *lacZ* genes were coexpressed to form a fusion protein. (E) Comparison of the influences of the *cre*<sub>P</sub> and *cre*<sub>ORF</sub> sites on CcpA expression. P(wt) and P(m), wild-type and *cre*<sub>P</sub> mutant promoter region sequences of *ccpA*, respectively. *ccpA*(wt) and *ccpA*(m), wild-type and *cre*<sub>ORF</sub> mutant *ccpA* genes, respectively. All data from the *in vivo* assay represent averages from two independent samples.

sites, respectively, were obtained by using overlapping PCR. These two mutated DNA fragments (*cre*<sub>P</sub>-m and *cre*<sub>ORF</sub>-m) and the corresponding original sequences (*cre*<sub>P</sub> and *cre*<sub>ORF</sub>) were then separately linked to the reporter gene *lacZ*, yielding a series of expression plasmids for *in vivo* β-galactosidase activity assays.

A 5′ rapid amplification of cDNA ends (5′-RACE) experiment showed that $cre_P$ is located upstream of the −35 region of the $ccpA$ promoter (Fig. 4B). The mutation of $cre_P$ (by replacing TGTTATATAACA with GGGGATATAACA) resulted in an approximately 50% decrease in LacZ activity from that of the original construct in wild-type *C. acetobutylicum*, whereas these types of mutations did not significantly change LacZ activity in *ccpA*-inactivated *C. acetobutylicum* (Fig. 4C). This result further verified CcpA-$cre_P$ binding activity *in vivo* and also suggested that the binding of CcpA to $cre_P$ can promote the expression of CcpA. A following question is whether the CcpA-$cre_P$ binding has a phenotypic effect on *C. acetobutylicum*. Thus, the plasmid-carried $ccpA$ gene, under the control of its native or $cre_P$-mutated (by replacing TGTTATATAACA with GGGGATATA ACA) promoter, was introduced into the above-described *ccpA*-inactivated *C. acetobutylicum* mutant for expression. The resulting strains, 824m-P-ccpA and 824m-Pm-ccpA, were compared to see the phenotypic changes. As shown in Fig. S1 in the supplemental material, the mutation of the $cre_P$ site caused an impaired cellular performance in fermentation using glucose as the sole carbon source. Therefore, it seems that the transcriptional autoactivation of CcpA by CcpA-$cre_P$ binding plays an important role in the metabolic regulation of *C. acetobutylicum*.

For the $cre_{ORF}$ site, its mutation (by replacing AAGAAAAAGATTACAT with AGGAGA AGGACTATAT) yielded LacZ activity that was approximately 3-fold greater than that of the original sequence in the wild-type *C. acetobutylicum* (Fig. 4D); no significant change resulted from the mutation of $cre_{ORF}$ in *ccpA*-inactivated *C. acetobutylicum* (Fig. 4D). These results also validated *in vivo* CcpA-$cre_{ORF}$ binding, but in contrast to the case for $cre_P$, the CcpA-$cre_{ORF}$ binding represses $ccpA$ expression (Fig. 4D).

Next, both $cre_P$ and $cre_{ORF}$ were mutated to examine whether they caused changes in $ccpA$ expression *in vivo*. As shown in Fig. 4E, the double mutations led to increased LacZ activities before 48 h; in contrast, LacZ activity in the mutated and the original constructs did not differ after 60 h. This result indicates that $cre_{ORF}$-based repression plays a more dominant role than $cre_P$-based activation in CcpA autoregulation during the early and middle cultivation phases; in the late phase, these two-way autoregulations based on $cre_P$ and $cre_{ORF}$ tend to balance each other.

Since CcpA can bind to both $cre_P$ and $cre_{ORF}$, a question that arises is whether the CcpA binding affinities for these two sites differ. To explore this possibility, competitive EMSAs were performed in which four DNA fragments, namely, Cy5-labeled DNA fragments that contained either $cre_P$ or $cre_{ORF}$ (Cy5-Df1 and Cy5-Df2) and nonlabeled DNA fragments that contained either $cre_P$ or $cre_{ORF}$ (Df1 and Df2), were used as probes. The results show that CcpA-$cre_{ORF}$ binding was almost completely abolished in the presence of 25- to 100-fold-higher concentrations of Df1 (Fig. 5A); 25- to 100-fold-higher concentrations of nonlabeled DNA fragment Df2 (the specific competitor) were used as the positive control for the competitive EMSAs (Fig. 5A). In contrast, CcpA-$cre_P$ binding was less affected by 25- to 100-fold-higher concentrations of Df2 (Fig. 5B); the 25- to 100-fold-higher concentrations of nonlabeled Df1 were adopted here as the positive control in the competitive EMSAs (Fig. 5B). Together, these results suggested that CcpA has a higher binding affinity to $cre_P$ than to $cre_{ORF}$.

**Interference with CcpA autoregulation improves cellular performance.** CcpA is able to control its own expression, but it is unknown what phenotypic changes will occur if $ccpA$ expression is free from this autoregulation. As a preliminary test, we overexpressed the $ccpA$ gene under the control of a widely used constitutive $P_{thl}$ promoter (the promoter of gene CAC2873) in *C. acetobutylicum*, which is not affected by CcpA (unpublished data). Interestingly, this design led to significantly improved cellular performance using glucose as the sole carbon source, i.e., higher growth rate and glucose consumption, greater acid reassimilation, and greater solvent formation (Fig. 6). After 72 h of fermentation, the $ccpA$-overexpressing strain 824-ccpA produced 21.98 g/liter of total solvents (5.71 g/liter acetone, 2.88 g/liter ethanol, and 13.39 g/liter butanol), approximately 15% higher than that (19.11 g/liter) of the control strain (824-P). Next, a quantitative reverse transcription-PCR (qRT-PCR) experiment was per-

(A)



(B)
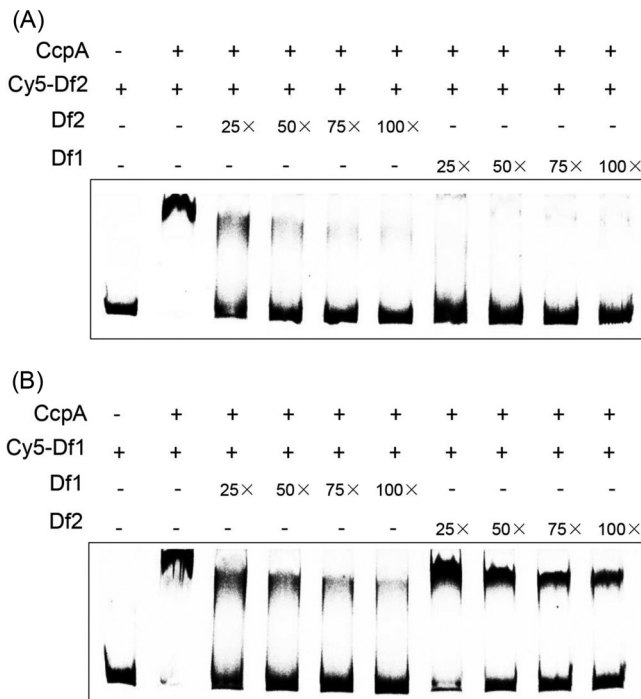


**FIG 5** Competitive EMSAs for calculation of binding affinities of the CcpA protein with the $cre_P$ and $cre_{ORF}$ sites. (A) Competitive effect of $cre_P$ on CcpA-$cre_{ORF}$ binding. (B) Competitive effect of $cre_{ORF}$ on CcpA-$cre_P$ binding. The initial concentration of Cy5-Df1 and Cy5-Df2 DNA was 0.04 pM, and 1.0 $\mu$M CcpA was used.

formed to examine the difference in *ccpA* transcription levels between the wild-type and *ccpA*-overexpressing strains, and as expected, the results confirmed successful plasmid-borne *ccpA* overexpression that led to a significant increase in *ccpA* transcription (Table 1). These results demonstrate that enhanced CcpA expression can positively change the phenotype of *C. acetobutylicum*.

However, the CcpA-binding $cre_{ORF}$ site in the coding region still existed in the above-described *ccpA*-overexpressing construct and could somewhat repress *ccpA* expression. To assess this problem, a synonymous mutation was made in $cre_{ORF}$, namely, replacing AAGAAAAAGATTACAT with AGGAGAAGGACTATAT. The *ccpA* gene harboring the mutated $cre_{ORF}$ sequence was then introduced into the wild-type strain for overexpression, yielding a new variant, 824-ccpA-m. In the fermentation using glucose as the sole carbon source, the growth, sugar consumption rate, and solvent synthesis of the 824-ccpA-m strain were, as expected, even better than those of 824-ccpA (the first *ccpA*-overexpressing strain mentioned above), producing 24.48 g/liter total solvents (6.55 g/liter acetone, 3.36 g/liter ethanol, and 14.57 g/liter butanol), which is 28% higher than the level for strain 824-P (Fig. 6). This result indicates that the synonymous $cre_{ORF}$ mutation might relieve the binding of CcpA to this site, thus eliminating CcpA self-repression and releasing more CcpA protein to exert its regulatory function.

The utilizations of xylose and a glucose-xylose mixture by the three strains described above were also compared. In the fermentation using xylose as the sole carbon source, both 824-ccpA and 824-ccpA-m behaved worse than the control strain 824-P, showing slower growth, sugar consumption, and formation of solvents (see Fig. S2 in the supplemental material). These phenotypic changes should be attributed to the enhanced carbon catabolite repression (CCR) that is caused by intracellular overexpression of *ccpA*. Hence, when using the mixture of glucose and xylose, compared to 824-P, both 824-ccpA and 824-ccpA-m showed slightly decreased xylose utilization, although they exhibited faster growth and glucose consumption (see Fig. S3 in the supplemental material). This is consistent with the phenotypic changes of 824-ccpA and 824-ccpA-m
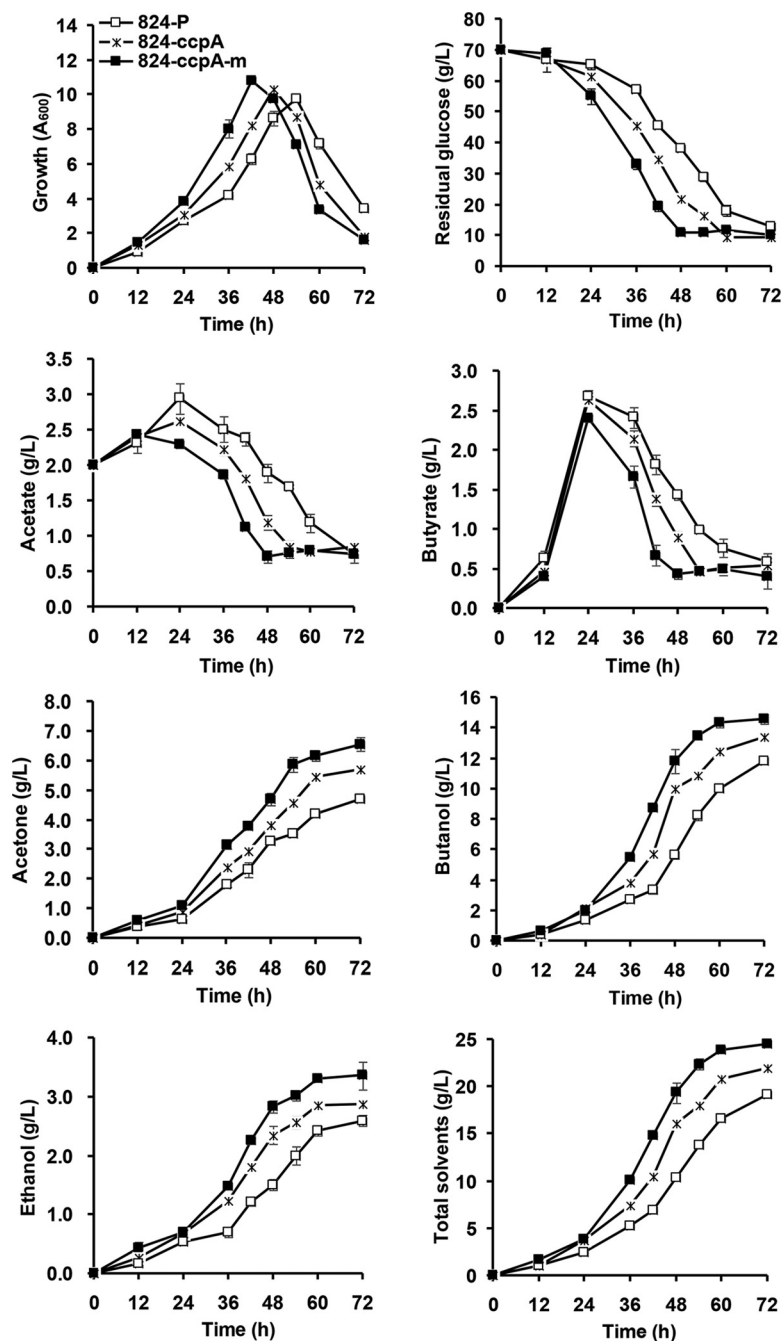
**FIG 6** Fermentation profiles of the *ccpA*-overexpressing strains and the wild-type strain using glucose as the sole carbon resource. 824-P, wild-type *C. acetobutylicum* strain harboring the control plasmid pIMP1-P$_{thl}$. 824-ccpA, *C. acetobutylicum* strain harboring plasmid pIMP1-P$_{thl}$-ccpA, which carries a wild-type *ccpA* gene for overexpression. 824-ccpA-m, *C. acetobutylicum* strain harboring plasmid pIMP1-P$_{thl}$-ccpA$_{mut}$, which carries the *cre*$_{ORF}$ mutant *ccpA* gene for overexpression. Total solvents represent the sum of acetone, butanol, and ethanol. The mean from three independent biological replicates and the standard deviation are shown.

in total solvent production compared to 824-P, namely, a higher production rate but slightly lower final titer (Fig. S3).

## DISCUSSION

As an important global regulator in Gram-positive bacteria, CcpA directly or indirectly regulates many genes. Thus, the CcpA-mediated regulatory system is always

**TABLE 1** Transcriptional changes of the *ccpA* gene after *ccpA* overexpression in *C. acetobutylicum*

| Strain[a] | Fold change in *ccpA* transcription (mean ± SD) after the following fermentation time (h): | | |
| --- | --- | --- | --- |
| | 24 | 48 | 72 |
| 824-P | 1 | 1.28 ± 0.26 | 0.53 ± 0.04 |
| 824-CcpA | 3,879.7 ± 188.1 | 2,767.7 ± 398.2 | 1,029.2 ± 198.6 |

[a]824-P, wild-type *C. acetobutylicum* strain harboring the control plasmid pIMP1-P$_{thl}$; 824-CcpA, the engineered *C. acetobutylicum* strain harboring the *ccpA*-overexpressing plasmid pIMP1-P$_{thl}$-ccpA (Table 2).

associated with many important cellular phenotypes (20–23). In light of the data here, we propose an autoregulation mechanism of CcpA based on a novel dual-*cre* motif in *C. acetobutylicum*, a typical species of solventogenic clostridia. This model includes two CcpA-binding sites: CcpA-*cre*$_P$ binding, which occurs within the promoter region, promotes *ccpA* transcription, whereas CcpA-*cre*$_{ORF}$ binding, which occurs within the coding region, leads to transcriptional repression. Importantly, we verified that CcpA autoregulation is essential to physiological and metabolic processes of *C. acetobutylicum*, indicating its significance in the regulatory network.

The binding of CcpA to *cre* sites in gene coding regions or the regions downstream of promoters generally leads to transcriptional repression, whereas binding in the regions upstream of promoters activates transcription (24). The self-promotion of CcpA expression via the *cre*$_P$ site should therefore be due to the relative position of *cre*$_P$ in the promoter region, i.e., the area upstream of the −35 region. This positioning allows CcpA to bind at this location and then recruit RNA polymerase to activate and enhance the transcription of *ccpA* (25, 26). Here, the 12-bp *cre*$_P$ (TGTTATATAACA) site shares low homology with the typical *cre* motif as well as the newly reported flexible *cre*$_{var}$ architecture in *C. acetobutylicum* (15, 19). This explains why the *cre*$_P$ site was missed in the previous screening, which used the typical *cre* motif (15).

Although both *cre*$_P$ and *cre*$_{ORF}$ were bound by CcpA, the former exhibited higher *in vitro* binding affinity to CcpA (Fig. 5). The higher binding affinity may establish an advantage for *cre*$_P$ over *cre*$_{ORF}$ in binding to CcpA when the intracellular CcpA level is still low during the early stages of the cell life cycle, thereby enabling rapid initiation of CcpA expression as well as the whole CcpA regulatory network. However, although *cre*$_P$ bound more strongly than *cre*$_{ORF}$ to CcpA, *cre*$_P$-based CcpA self-promotion was unexpectedly weaker in intensity than *cre*$_{ORF}$-based self-repression during most of the culturing time (Fig. 4E). This result indicates that CcpA autoregulation in *C. acetobutylicum* is more strongly influenced by the "roadblock" effect in the coding region that results from CcpA binding to the *cre*$_{ORF}$ site.

Most TFs identified in prokaryotic microorganisms employ one binding site to achieve their autoregulation; even in the few autoregulation cases involving multiple binding sites, these *cis* elements nearly always occur in the promoter region (5, 7, 27–29). Specifically considering the global regulator CcpA, only *Lactobacillus pentosus* and *Staphylococcus xylosus* putative *cre* sites were predicted to be present in the promoter region of *ccpA* (17, 18), indicating a potential autoregulation mechanism that has yet to be experimentally verified. Thus, the dual-*cre* motif discovered in this study revealed the first appearance of a novel two-way autoregulation mode for CcpA in Gram-positive bacteria. Since the two *cre* sites (*cre*$_P$ and *cre*$_{ORF}$) in this model bound CcpA with different strength, it can be speculated that such a dual-*cre* model enables more flexible regulation than the single-*cre* form.

It should be noted that *cre*$_{ORF}$ is actually a dual-use codon (i.e., not only a regulatory site but also a region encoding amino acids). Such dual-use codons occur frequently in many microorganisms but are rarely involved in autoregulation. We are still unclear whether *cre*$_{ORF}$-like TF-binding sites within exons have greater or special significance in autoregulation compared to typical binding sites in promoter regions, and to find an answer requires extensive exploration by studying more examples.

In the case of CcpA-dependent carbon catabolite activation (CCA) in Gram-positive bacteria, our understanding is limited to its role in activating gene transcription, which is often caused by the interaction of the complex of P-Ser-HPr/P-Ser-Crh and CcpA with RNA polymerase (30). For example, CcpA can directly or indirectly activate the transcription of several essential genes involved in central carbon catabolism (31–33) and amino acid synthesis pathways (34, 35) that are crucial for maintaining cellular viability. However, in this study, enhanced CcpA expression levels could lead to improved cell performance in fermenting glucose. This improvement may be attributed to the reinforcement of CCA by overexpression of the *ccpA* gene, leading to the transcriptional upregulation of some relevant genes. However, the enhanced CcpA level caused by *ccpA* overexpression also had negative effects, namely, strengthening of its repression of the metabolic pathways of some nonpreferable sugars (e.g., xylose here) and then inhibiting the use of these sugars. To further address this problem, identification and mutation of CcpA-binding sites surrounding the essential genes in the pathways will be a useful strategy (15). In conclusion, the results here indicate that the CcpA regulatory network in *C. acetobutylicum*, as well as other Gram-positive bacteria, may be optimized further.

Here, we have discovered a novel CcpA autoregulation mechanism in *C. acetobutylicum*, which is based on a previously unreported dual-*cre* motif. The two *cre* sites in this motif reside in the promoter and coding regions for CcpA and have two opposite effects on CcpA autoregulation. This two-way autoregulation mechanism may enable CcpA to more effectively control and modulate the downstream regulatory network and may simultaneously provide new insights into regulation patterns of global TFs. Finally, considering the phenotypic changes caused by changes in the intracellular CcpA level, modification of the CcpA autoregulation model may be valuable in future metabolic engineering applications.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, media, and growth conditions.** The strains and plasmids used in this work are listed in Table 2. *E. coli* strains DH5$\alpha$ and ER2275 were grown in Luria-Bertani (LB) medium or on LB agar. *E. coli* strain Rosetta(DE3) was used as the host for protein purification. LB medium was supplemented with appropriate concentrations of antibiotics (100 $\mu$g/ml ampicillin and 100 $\mu$g/ml spectinomycin) when needed. The wild-type *C. acetobutylicum* strain ATCC 824 was maintained in 75% glycerol and stored at −70°C. The *C. acetobutylicum ccpA*-disrupted mutant was constructed previously by using the ClosTron system (14). The *in vivo* methylation of plasmids was performed in *E. coli* strain ER2275, and they were then transformed into *C. acetobutylicum* strains through electroporation. CGM medium (36) and P2 medium (37) were used for inoculum preparation and fermentation of *C. acetobutylicum* strains, respectively. Erythromycin (10 $\mu$g/ml) was added to P2 medium when needed.

**Plasmid construction.** The plasmids and primers used in this work are listed in Table 2 and in Table S1 in the supplemental material, respectively. For *ccpA* overexpression, the promoter P$_{thl}$ was first PCR amplified from the plasmid pIMP1-P$_{thl}$ by using primers P$_{thl}$-PstI-for/P$_{thl}$-ccpA-rev and then linked to the *ccpA* gene via overlapping PCR by using primers ccpA-P$_{thl}$-for/ccpA-EcoRI-rev. The resulting PCR fragment was digested with PstI/EcoRI and then inserted into plasmid pIMP1, which was digested with the same restriction enzymes, yielding plasmid pIMP1-P$_{thl}$-ccpA. Plasmid pIMP1-P$_{ccpAmut}$-ccpA, containing *cre*$_P$-m, was constructed by using primers P$_{ccpA}$-PstI-for/P$_{ccpAmut}$-rev and P$_{ccpAmut}$-for/ccpA-EcoRI-rev, two fragments were linked via overlapping PCR, and then it was digested with PstI/EcoRI and inserted into plasmid pIMP1.

The construction of plasmid pIMP1-P$_{ccpA}$-ccpA-lacZ was performed as follows. A DNA fragment containing the promoter region and part of the coding region (from nucleotide −244 to +372) of the *ccpA* gene was PCR amplified from the genome of *C. acetobutylicum* ATCC 824 by using primers P$_{ccpA}$-PstI-for/O$_{ccpA}$-lacZ-rev and then linked to the *lacZ* reporter gene via overlapping PCR by using primers lacZ-O$_{ccpA}$-for/lacZ-SmaI-rev. The resulting DNA fragment was digested with PstI/SmaI and then inserted into plasmid pIMP1, which was digested with the same restriction enzymes, yielding plasmid pIMP1-P$_{ccpA}$-ccpA-lacZ.

The construction of the other derivative plasmid (pIMP1-P$_{ccpAmut}$-ccpA$_{mut}$-lacZ) was similar to the protocol described above except that the primers (P$_{ccpAmut}$-for/P$_{ccpAmut}$-rev and ccpA$_{mut}$-for/ccpA$_{mut}$-rev) used for overlapping PCR contained mutated *cre*$_P$ and *cre*$_{ORF}$ sites.

**β-Galactosidase assay.** The plasmids carrying the *lacZ* gene were transformed into the *C. acetobutylicum* wild-type strain and derivative mutants. Cells were grown anaerobically in P2 medium (containing 70 g/liter glucose) at 37°C for 24 h or 48 h, reaching an optical density at 600 nm (OD$_{600}$) of 3 or 7, respectively. Pellets of 5 ml of grown cells were collected by centrifugation at 5,000 × *g* for 10 min at 4°C and then resuspended in 300 $\mu$l B-PER reagent (Thermo Scientific Pierce) and vortexed for 1 min. The cell lysate was heat treated at 60°C for 30 min, and the precipitate was removed by centrifugation at

**TABLE 2** Strains and plasmids used in this study

| Strain or plasmid | Description | Source or reference |
|---|---|---|
| **Bacterial strains** | | |
| *C. acetobutylicum* | | |
| 824 | ATCC 824, wild-type strain | ATCC |
| ΔccpA | *ccpA*::intron | 14 |
| 824-P | ATCC 824 carrying the pIMP1-P$_{thl}$ plasmid | This study |
| 824-ccpA | ATCC 824 carrying the pIMP1-P$_{thl}$-ccpA plasmid | This study |
| 824-ccpA-m | ATCC 824 carrying the pIMP1-P$_{thl}$-ccpA$_{mut}$ plasmid | This study |
| 824 m-P-ccpA | ΔccpA carrying the pIMP1-P$_{ccpA}$-ccpA plasmid | This study |
| 824 m-Pm-ccpA | ΔccpA carrying the pIMP1-P$_{ccpAmut}$-ccpA plasmid | This study |
| cre$_P$/824 | ATCC 824 carrying the pIMP1-P$_{ccpA}$-lacZ plasmid | This study |
| cre$_P$-m/824 | ATCC 824 carrying the pIMP1-P$_{ccpAmut}$-lacZ plasmid | This study |
| cre$_{ORF}$/824 | ATCC 824 carrying the pIMP1-P$_{ccpA}$-ccpA-lacZ plasmid | This study |
| cre$_{ORF}$-m/824 | ATCC 824 carrying the pIMP1-P$_{ccpA}$-ccpA$_{mut}$-lacZ plasmid | This study |
| cre$_{ORF}$/ΔccpA | ΔccpA carrying the pIMP1-P$_{ccpA}$-ccpA-lacZ plasmid | This study |
| cre$_{ORF}$-m/ΔccpA | ΔccpA carrying the pIMP1-P$_{ccpA}$-ccpA$_{mut}$-lacZ plasmid | This study |
| *E. coli* | | |
| Top10 | General cloning host strain | Invitrogen |
| ER2275 | Strain used to methylate the expression vector | New England Biolabs |
| Rosetta(DE3) | Strain used for protein overexpression | Novagen |
| | | |
| **Plasmids** | | |
| pIMP1-P$_{thl}$ | Vector with *thl* promoter | This study |
| pIMP1-P$_{thl}$-ccpA | *ccpA* overexpression vector derived from pIMP1-P$_{thl}$ | This study |
| pIMP1-P$_{thl}$-ccpA$_{mut}$ | *ccpA$_{mut}$* overexpression vector derived from pIMP1-P$_{thl}$ | This study |
| pIMP1-P$_{ccpA}$-ccpA | *ccpA* overexpression vector derived from pIMP1-P$_{ccpA}$ | This study |
| pIMP1-P$_{ccpAmut}$-ccpA | *ccpA* overexpression vector derived from pIMP1-P$_{ccpAmut}$ | This study |
| pIMP1-P$_{ccpA}$-lacZ | *lacZ* reporter gene driven by *ccpA* promoter | This study |
| pIMP1-P$_{ccpAmut}$-lacZ | *lacZ* reporter gene driven by mutant *ccpA* promoter | This study |
| pIMP1-P$_{ccpA}$-ccpA-lacZ | Fusion of a segment of the *ccpA* gene and *lacZ* reporter driven by the *ccpA* promoter | This study |
| pIMP1-P$_{ccpA}$-ccpA$_{mut}$-lacZ | Fusion of a segment of the *ccpA$_{mut}$* gene and *lacZ* reporter driven by the *ccpA* promoter | This study |
| pIMP1-P$_{ccpAmut}$-ccpA$_{mut}$-lacZ | Fusion of a segment of the *ccpA$_{mut}$* gene and *lacZ* reporter driven by the *ccpA$_{mut}$* promoter | This study |

12,000 × *g* for 30 min. The supernatant was used for LacZ (*β*-galactosidase) activity assay according to a protocol reported previously (38).

**Protein overexpression and purification.** To purify the CcpA protein, the *ccpA* gene (CAC3037) of *C. acetobutylicum* ATCC 824 was PCR amplified and then inserted into plasmid pET-28a (Invitrogen). The resulting plasmid, pET-28a-ccpAcac, was transformed into the *E. coli* Rosetta(DE3) strain for protein expression. The subsequent purification procedure was the same as previously described (15).

**EMSA.** The probes used for electrophoretic mobility shift assay (EMSA) were generated by two-step PCR amplification. First, the unlabeled DNA fragments were PCR amplified from the *C. acetobutylicum* genome using specific primer pairs containing a universal sequence (5′-AGCCAGTGGCGATAAG-3′) at the 5′ terminus. Next, the labeled DNA fragments were obtained by PCR using a 5′-Cy5-labeled universal primer, 5′-AGCCAGTGGCGATAAG-3′, and then recovered by agarose gel electrophoresis as probes for EMSAs. The EMSAs were performed as previously described (15). The voltage used in EMSA was 110 V.

**Real-time qRT-PCR.** The *C. acetobutylicum* cells were grown in P2 medium using D-glucose (70 g/liter) as the sole carbon source. Samples were harvested at three time points (24, 48, and 72 h). Total RNA was extracted with a kit (catalog number cw0581; CWbio) according to the manufacturer's instructions. Contaminating DNA was eliminated by DNase I (TaKaRa) digestion, which was verified by PCR analysis using the RNA as the template. The concentration of RNA was determined by using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). cDNA was obtained by reverse transcription using the PrimeScript RT reagent kit (TaKaRa). Real-time quantitative reverse transcription-PCRs (qRT-PCRs) were preformed in a MyiQ2 real-time PCR detection system (Bio-Rad). The reaction mixtures (20 *μ*l) contained 1× iQ SYBR green Supermix (Bio-Rad), 0.5 *μ*M each primer, and the diluted cDNA template (0.625 ng/*μ*l), and the reaction conditions were as follows: an initial denaturation at 95°C for 10 min, 40 cycles of 15 s at 95°C, and a final incubation at 60°C for 1 min. The primers used for qRT-PCR are listed in Table S1. The CAC2679 gene was used as the internal control.

**5′-RACE.** The transcriptional start site of the *ccpA* gene was determined by the 5′ rapid amplification of cDNA ends (5′-RACE) method. *C. acetobutylicum* was cultivated in P2 medium using D-glucose (70 g/liter) as the carbon source, and the samples were harvested at 24 and 48 h. Total RNA was isolated by using a TRIzol (Invitrogen) extraction kit. The 5′-RACE experiment was performed using a 5′-full RACE kit with TAP (catalog number 6107; TaKaRa). Primers ccpA-outer-Dw/ccpA-inner-Dw were used for PCR by the cDNA fragment of *ccpA* as the template. Finally, the PCR products were cloned into T-vector for sequencing.

**Fermentation.** Inoculum preparation and fermentations of *C. acetobutylicum* were performed anaerobically in CGM and P2 media, respectively, to which erythromycin (10 *μ*g/ml) was added when needed. The detailed manipulations were similar to those previously described (39). In brief, 150 *μ*l

frozen stock of *C. acetobutylicum* was transferred into 5 ml liquid CGM medium and then incubated anaerobically at 37°C. When the $OD_{600}$ of grown cells reached 0.8 to 1.0, 2.5 ml of the grown cells was inoculated into 50 ml liquid P2 medium for fermentation. In the fermentations, D-glucose (70 g/liter), D-xylose (60 g/liter), or a mixture of D-glucose and D-xylose (40 g/liter glucose and 20 g/liter xylose) was used as the carbon source.

**Analytical methods.** Cell growth was monitored based on the $OD_{600}$ of the culture using a spectrophotometer (DU730; Beckman Coulter). For analysis of fermentation products (acetone, acetic acid, butyric acid, butanol, and ethanol) and sugars (glucose and xylose), samples were taken at appropriate time intervals and then centrifuged at $7,000 \times g$ for 10 min at 4°C. The supernatant was then analyzed. The concentrations of fermentation products were determined by gas chromatography (7890A; Agilent, Wilmington, DE, USA) equipped with a capillary column (Alltech EC-Wax) and a flame ionization detector (Agilent). The glucose and xylose concentrations were measured with a high-pressure liquid chromatography system (model 1200; Agilent) equipped with a Sugar-PakTMI column (Waters Corp., MA, USA) and a refractive index detector (Agilent). The analyses of fermentation products and sugars were carried out as described previously (14).

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM .00114-18.

**SUPPLEMENTAL FILE 1,** PDF file, 0.1 MB.

## ACKNOWLEDGMENTS

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