AraR, an L-Arabinose-Responsive Transcriptional Regulator in *Corynebacterium glutamicum* ATCC 31831, Exerts Different Degrees of Repression Depending on the Location of Its Binding Sites within the Three Target Promoter Regions

Takayuki Kuge,^{a,b} Haruhiko Teramoto,^a Masayuki Inui^{a,b}

Research Institute of Innovative Technology for the Earth, Kizugawa, Kyoto, Japan^a; Graduate School of Biological Sciences, Nara Institute of Science and Technology, Takayama, Ikoma, Nara, Japan^b

ABSTRACT

In *Corynebacterium glutamicum* ATCC 31831, a LacI-type transcriptional regulator AraR, represses the expression of L-arabinose catabolism (*araBDA*), uptake (*araE*), and the regulator (*araR*) genes clustered on the chromosome. AraR binds to three sites: one (BS_B) between the divergent operons (*araBDA* and *galM-araR*) and two (BS_{E1} and BS_{E2}) upstream of *araE*. L-Arabinose acts as an inducer of the AraR-mediated regulation. Here, we examined the roles of these AraR-binding sites in the expression of the AraR regulon. BS_B mutation resulted in derepression of both *araBDA* and *galM-araR* operons. The effects of BS_{E1} and/or BS_{E2} mutation on *araE* expression revealed that the two sites independently function as the *cis* elements, but BS_{E1} plays the primary role. However, AraR was shown to bind to these sites with almost the same affinity *in vitro*. Taken together, the expression of *araBDA* and *araE* is strongly repressed by binding of AraR to a single site immediately downstream of the respective transcriptional start sites, whereas the binding site overlapping the −10 or −35 region of the *galM-araR* and *araE* promoters is less effective in repression. Furthermore, downregulation of *araBDA* and *araE* dependent on L-arabinose catabolism observed in the BS_B mutant and the AraR-independent *araR* promoter identified within *galM-araR* add complexity to regulation of the AraR regulon derepressed by L-arabinose.

IMPORTANCE

Corynebacterium glutamicum has a long history as an industrial workhorse for large-scale production of amino acids. An important aspect of industrial microorganisms is the utilization of the broad range of sugars for cell growth and production process. Most *C. glutamicum* strains are unable to use a pentose sugar L-arabinose as a carbon source. However, genes for L-arabinose utilization and its regulation have been recently identified in *C. glutamicum* ATCC 31831. This study elucidates the roles of the multiple binding sites of the transcriptional repressor AraR in the derepression by L-arabinose and thereby highlights the complex regulatory feedback loops in combination with L-arabinose catabolism-dependent repression of the AraR regulon in an AraR-independent manner.

Lignocellulosic biomass from agricultural and agro-industrial residues represents one of the important renewable resources expected to be used for the industrial production of biofuels and biochemicals (1, 2). However, significant amounts of pentose sugars derived from its hemicellulose component are one major technical hurdle in the use for economically feasible bioprocesses. These sugars, such as D-xylose and L-arabinose, are not efficiently utilized by conventional and industrial microorganisms, and improvement of the pentose sugar metabolic pathways by genetic engineering is often limited by preferential utilization of a most abundant and favorable hexose sugar, D-glucose, in lignocellulosic biomass (3).

Corynebacterium glutamicum is a Gram-positive actinobacterium with a high G+C content in its genomic DNA. It has a long history as an industrial workhorse for large-scale production of amino acids (e.g., L-glutamate and L-lysine) (4, 5). Moreover, investigation into its application in the production of fuels and commodity chemicals is beginning to be productive (6–8). Recent advances in metabolic engineering of *C. glutamicum* open up new possibilities for efficient utilization of substrates containing mixtures of D-glucose, D-xylose, and L-arabinose (9–12). Understanding of the unique transcriptional regulatory system of sugar me-

tabolism genes in *C. glutamicum* (13, 14) highlights a potential avenue toward optimization of utilization of sugar mixtures derived from a variety of lignocellulosic feedstocks.

Generally, L-arabinose taken up by cells via transporters is sequentially converted to L-ribulose, L-ribulose 5-phosphate, and D-xylulose 5-phosphate by the action of L-arabinose isomerase (encoded by *araA*), L-ribulokinase (encoded by *araB*), and L-ribulose 5-phosphate 4-epimerase (encoded by *araD*), respectively.

Received 5 July 2015 Accepted 21 September 2015

Accepted manuscript posted online 28 September 2015

Citation Kuge T, Teramoto H, Inui M. 2015. AraR, an L-arabinose-responsive transcriptional regulator in *Corynebacterium glutamicum* ATCC 31831, exerts different degrees of repression depending on the location of its binding sites within the three target promoter regions. J Bacteriol 197:3788–3796. doi:10.1128/JB.00314-15.

Editor: I. B. Zhulin

Address correspondence to Masayuki Inui, mmg-lab@rite.or.jp.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/JB.00314-15>.

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FIG 1 Arabinose gene cluster in *C. glutamicum* ATCC 31831. (A) Location and direction of L-arabinose utilization genes (*araA*, *araB*, *araD*, *araE*, *araR*, and *galM*). The amplified fragments in Fig. 1D are indicated by numbers and lines. (B and C) Nucleotide sequences of the *araB*-*galM* intergenic region (B) and the *araE* upstream region (C). The translation start codons are boxed, and the promoter elements, such as the -10 and -35 motifs, based on the transcription start sites which are indicated by bending arrows, are indicated by asterisks. Three AraR-binding sites in the *galM/araB* and *araE* promoter regions (BS_B, BS_{E1}, and BS_{E2}) are shaded. Substituted nucleotides in AraR binding site mutants are shown below the respective native sequences. (D) Agarose gel electrophoresis of the RT-PCR products. The genes corresponding to the primers used are indicated above each pair of lanes; the same preparations of *C. glutamicum* genomic DNA (left-hand lane), total RNA (middle lane), and cDNA (right-hand lane) were used as the templates. The primers (see Table S2 in the supplemental material) are as follows: 1-F/1-R (lanes 1), 2-F/2-R (lanes 2), 3-F/3-R (lanes 3), 4-F/4-R (lanes 4), 5-F/5-R (lanes 5), 6-F/6-R (lanes 6). M, DNA size marker.

D-Xylulose 5-phosphate is further catabolized through the pentose phosphate pathway. *Escherichia coli* AraC (15) and *Bacillus subtilis* AraR (16, 17) are well-characterized transcriptional regulators of the L-arabinose utilization genes. Although these regulators belong to distinct families, the two are directly involved in strong upregulation of L-arabinose catabolic and uptake genes but in weak upregulation of their own genes in response to L-arabinose (18–20). Cooperative binding of the respective regulator proteins to multiple sites within each of their target promoters plays a critical role in the tight regulation.

Although most *C. glutamicum* strains are unable to use L-arabinose as a carbon source, *C. glutamicum* ATCC 31831 has its utilization ability due to the presence of *araBDA* on its genome

(21). We have recently shown that a LacI family transcriptional regulator AraR, which is encoded in the vicinity of the L-arabinose catabolic genes (*araBDA*), along with its uptake gene (*araE*) on the genome, binds *in vitro* to three sites: one in the intergenic region of *araB/galM* (BS_B) and two upstream of *araE* (BS_{E1}/BS_{E2}) (Fig. 1). Based on the sequences of the N-terminal helix-turn-helix DNA-binding domains, *C. glutamicum* ATCC 31831 AraR and *B. subtilis* AraR proteins are assigned to distinct families: the LacI family and the GntR family, respectively (22, 23). However, their C-terminal effector-binding domains are homologous to the LacI family proteins. DNA binding activity of the *C. glutamicum* protein is indicated to be decreased in the presence of L-arabinose, resulting in derepression of *araBDA* and *araE* (24). A slight increase in the

expression levels of *galM* and *araR* in response to L-arabinose suggests that AraR also regulates the transcription of *galM-araR* as an operon. However, the effects of L-arabinose supplementation or *araR* inactivation on the expression of *galM-araR* are much smaller than those on the expression of the divergently transcribed *araBDA*, the regulatory mechanism for which remains unclear. On the other hand, the AraR protein is suspected to exert cooperative interactions with the two sites located within the *araE* promoter region, as in the case of the tight regulation by *E. coli* AraC and *B. subtilis* AraR. However, the role of the two AraR-binding sites in *araE* expression has not been verified experimentally.

In this study, we examine the roles of the three AraR-binding sites located in the *C. glutamicum* ATCC 31831 arabinose gene cluster in the AraR-mediated regulation in response to L-arabinose. As a result, an AraR-binding site (BS_B) within the *araB-galM* intergenic region is shown to be involved in the L-arabinose-responsive upregulation of not only the *araBDA* operon but also the *galM-araR* operon. We also show that the AraR protein independently binds to the two sites (BS_{E1} and BS_{E2}) upstream of *araE* *in vitro*. Analyses of mutant strains deficient in BS_{E1} and/or BS_{E2} indicate that binding of AraR to each site results in different degrees of repression of *araE* expression in the absence of L-arabinose. On the other hand, the L-arabinose utilization genes were downregulated in response to L-arabinose in BS_B mutant. The downregulation is indicated to be dependent on L-arabinose catabolism, suggesting that these genes are subject to the catabolic repression besides the substrate induction mediated by AraR.

MATERIALS AND METHODS

Bacterial strains, plasmids, oligonucleotides, and culture conditions. The strains and plasmids used in the present study are listed in Table S1 in the supplemental material. The oligonucleotide primers used are listed in Table S2 in the supplemental material. For genetic manipulation, *E. coli* strains were grown at 37°C in Luria-Bertani medium. *C. glutamicum* strains were grown at 33°C in nutrient-rich A medium [2% (wt/vol) yeast extract, 7% (wt/vol) Casamino Acids, 2% (wt/vol) (NH₂)₂CO, 7% (wt/vol) (NH₄)₂SO₄, 0.5% (wt/vol) KH₂PO₄, 0.5% (wt/vol) K₂HPO₄, 0.5% (wt/vol) MgSO₄·7H₂O, 0.006% (wt/vol) FeSO₄·H₂O, 0.0042% (wt/vol) MnSO₄·H₂O, 0.002% (wt/vol) biotin, 0.002% (wt/vol) thiamine]. *C. glutamicum* ATCC 31831 was used as a wild-type strain in the present study. The *araA* single deletion mutant strain derived from *C. glutamicum* ATCC 31831 wild-type strain (see Table S1 in the supplemental material; Δ araA) was described previously (24).

For analytical purposes, *C. glutamicum* starter culture was grown aerobically in 10 ml of nutrient-rich A medium in a test tube overnight. The cells were inoculated in fresh medium at a dilution of ≥ 100 -fold. The cells were cultured in 100 ml of nutrient-rich A medium at 33°C in a 500-ml flask. To assess the response to sugars, the medium was supplemented with L-arabinose at the stated concentrations.

RT-PCR. Total RNA was extracted from *C. glutamicum* cells using an RNeasy minikit (Qiagen) as described previously (24). For reverse transcription-PCR (RT-PCR) analysis, single-stranded cDNA was synthesized from total RNA using PrimeScript reverse transcriptase (TaKaRa) with hexadeoxyribonucleotide mixture as a primer. The resulting cDNA was used as a template for PCR with a primer set for the region of interest (see Table S2 in the supplemental material). *C. glutamicum* chromosomal DNA, prepared as described previously (24), was also used as a template for a control PCR with the same primer set. The reaction mixture was electrophoresed on an agarose gel, and the amplified DNA was detected with ethidium bromide.

Synthesized cDNA is also used as a template for quantitative RT-PCR analysis as described previously (24). The primers used are listed in Table S2 in the supplemental material. The relative abundance of the target

mRNAs was quantified based on the cycle threshold value. To standardize the results, the relative abundance of 16S rRNA was used as the internal standard.

Construction of a genetically modified strain. To modify the AraR binding site (BS_B, BS_{E1}, and BS_{E2}) on the chromosome, overlapping PCR was performed using genomic DNA as a template. The primer pairs used are summarized in Table S2 in the supplemental material. BSB-integ-FW/BSB-mut-RV and BSB-integ-RV/BSB-mut-FW were used for BS_B mutation, BSE-integ-FW/BSE1-mut-RV and BSE-integ-RV/BSE1-mut-FW were used for BS_{E1} mutation, and BSE-integ-FW/BSE2-mut-RV and BSE-integ-RV/BSE2-mut-FW were used for BS_{E2} mutation. The resulting fragments with base substitutions in the AraR binding site were digested with EcoRV-XbaI and cloned into pCRB206 (25), a suicide vector. The resulting plasmids, pCRF503, pCRF504, and pCRF505 were used for construction of the strains with base substitutions in BS_B, BS_{E1}, and BS_{E2}, respectively, as described previously (25). For mutagenesis of the two sites (BS_{E1} and BS_{E2}) simultaneously, overlapping PCR was performed using pCRF504 as a template and primers for BS_{E2} mutation as described above, yielding a plasmid pCRF506.

EMSA. AraR was expressed with an N-terminal His tag and purified by affinity chromatography as described previously (24). DNA probes were prepared by PCR from the *C. glutamicum* ATCC 31831 chromosomal DNA or the plasmids for construction of the strains with mutations in the AraR binding sites, as described above. Sets of primers used are ParaB-FW/ParaB-RV or ParaE-FW/ParaE-RV (see Table S2 in the supplemental material). Electrophoretic mobility shift assay (EMSA) was performed as described previously (24). The equilibrium dissociation constant (K_d) for the different operators was determined as the free concentration of AraR at which half molecules is bound. The concentration of free AraR protein was indirectly measured by subtracting the DNA-AraR concentration from the total AraR concentration.

Construction of the promoter-lacZ fusion. In order to integrate the promoter-reporter *lacZ* fusion genes into the *C. glutamicum* ATCC 31831 chromosome by homologous recombination, the upstream and downstream regions of the integration target site in a hypothetical gene about 2.5 kb downstream of *araA* were amplified using sets of primers summarized in Table S2 in the supplemental material (integ-top-FW/integ-top-DW and integ-bottom-Fw/integ-bottom-DW). The resultant amplicons were fused and cloned into the suicide vector pCRB206, yielding pCRF507. The *E. coli lacZ* gene was amplified using the oligonucleotide primers *lacZ*-FW and *lacZ*-DW (see Table S2 in the supplemental material) using pCRA741 (26) as a template. The 3.1-kb PCR product containing the *E. coli lacZ* was digested with ScaI and inserted into the unique ScaI site of pCRF507, yielding pCRF508 (see Fig. S3 in the supplemental material). A DNA fragment containing the *araR* or *galM* promoter region was amplified by PCR using *C. glutamicum* ATCC 31831 chromosomal DNA as a template and appropriate primers (see Table S2 in the supplemental material; ParaR-lacZ-FW/ParaR-lacZ-RV and PgalM-lacZ-FW/PgalM-lacZ-RV). The amplified DNA was digested with SpeI and inserted into the corresponding site of pCRF508. The sequence and direction of the cloned fragments were confirmed by the dideoxy chain termination method as described above. The resulting plasmid including the *araR* or *galM* promoter region in the canonical orientation relative to the *lacZ* gene was named pCRF509 or pCRF511, respectively, and the plasmid including the *araR* promoter region in the reverse orientation relative to the *lacZ* gene was named pCRF510 (see Table S1 in the supplemental material). These promoter-lacZ fusions were integrated into the *C. glutamicum* ATCC 31831 chromosome by markerless gene insertion methods described previously (25), yielding strains ParaR-F, PgalM-F, and ParaR-R. We confirmed that the integration of the *lacZ* fusion into the hypothetical gene has no effect on the L-arabinose-dependent regulation of the native *araBDA* genes.

β -Galactosidase assay. *C. glutamicum* cells were harvested, washed once with Z-buffer (27), resuspended with the same buffer, and treated with toluene. The permeabilized cells were then incubated with o-nitro-

phenyl- β -D-galactopyranoside, and the activity was measured in Miller units as described previously (27).

RESULTS

Transcriptional units of L-arabinose utilization genes. Previously, we reported that three L-arabinose catabolic genes, i.e., *araB*, *araD*, and *araA*, are similarly upregulated by L-arabinose supplementation or *araR* inactivation (24). However, whether they are cotranscribed as an operon was not addressed. The *araB-araD* and *araD-araA* intergenic regions are 13 and 35 bp long, respectively. Here, we tested whether the adjacent genes are cotranscribed by RT-PCR. As shown in Fig. 1D, a specific PCR product of *araB-araD* and *araD-araA* from cDNA amplified from exponentially growing cells cultured in the presence of L-arabinose (the third lane in each primer set), as well as from the chromosomal DNA (the first lane), were observed. In contrast, no product was amplified from total RNA without reverse transcription (the second lane), eliminating the possibility of chromosomal DNA contamination. As expected, we detect no RT-PCR product corresponding to the divergent *araB-galM* intervening region. These results confirm that *araD* and *araA* are cotranscribed with *araB* as the *araBDA* operon under the control of the *araB* promoter.

galM is located upstream of *araB* in the opposite direction with the 76-bp intergenic region. *araR* and *araE* are located downstream of *galM* and these three genes are transcribed in the same directions. *galM*, *araR*, and *araE* encode a putative aldose 1-epimerase, a LacI-type transcriptional regulator and L-arabinose/proton symporter, respectively. The *galM-araR* and *araR-araE* intergenic regions are 26 and 234 bp long, respectively. Previously, we detected a similar increase in the levels of *galM* and *araR* mRNAs in response to L-arabinose supplementation, but the degree was much smaller than in the case of *araE* mRNA (24). The expression pattern and the location of the *galM* and *araR* genes on the chromosome suggest that *araR* is cotranscribed with *galM* under the control of the L-arabinose-inducible promoter. However, we also observed upregulation of *araR* but not of *galM* in cells grown on L-arabinose compared to cells grown on D-glucose (21). Here, we detected the *galM-araR* intervening region amplified by RT-PCR performed as described above (Fig. 1B). In contrast, no RT-PCR product corresponding to the *araR-araE* intervening region was detected (data not shown). These results indicate that *araR* are cotranscribed with *galM* as the *galM-araR* operon under the control of the *galM* promoter.

In addition, to examine the possibility of *araR* expression under the control of its own promoter, a translational fusion of the 400-bp upstream region of *araR* to the *lacZ* reporter gene derived from *E. coli* was integrated into the *C. glutamicum* ATCC 31831 chromosome (Fig. 2A, *ParaR-F*). The reverse orientation of the same fragment was used as a negative control (Fig. 2A, *ParaR-R*). The β -galactosidase activity driven by the canonical and reverse promoters during growth in nutrient-rich A medium containing either 1% (wt/vol) D-glucose or 1% (wt/vol) L-arabinose was determined (Fig. 2A). In exponentially growing cells, β -galactosidase activity was detected in *ParaR-F* but not in *ParaR-R*. Moreover, no difference was observed in the *araR* promoter activity between the cells grown with D-glucose and the cells grown with L-arabinose. Next, to examine whether the L-arabinose-responsive upregulation of *araR* depends on the *galM* and/or *araR* promoters, we constructed a strain carrying the 400-bp *galM* promoter-

FIG 2 *galM* and *araR* promoter activities. (A) *araR* promoter activity. The 400-bp *araR* upstream region was fused to the *lacZ* reporter gene in the canonical (*ParaR-F*) or the reverse (*ParaR-R*) orientation. The *C. glutamicum* ATCC 31831 strains carrying the respective *lacZ* fusions in the chromosome were cultured in A medium containing either 2% (wt/vol) D-glucose or L-arabinose for 4 h, and the β -galactosidase activity was measured. The means $\pm$ the standard deviations of three independent experiments are shown. N.D., not detected. (B) Changes in the expression of the *galM* promoter-*lacZ* fusion (*PgalM-F*) and the *araR* promoter-*lacZ* fusion (*ParaR-F*) in response to L-arabinose. The strains carrying the respective *lacZ* fusions on the chromosome were grown in A medium for 4 h, and the exponentially growing cells were then supplemented with L-arabinose to a final concentration of 2% (wt/vol). The levels of *lacZ* mRNA in the cells incubated with L-arabinose for 0, 5, and 15 min were determined by qRT-PCR and are presented relative to the value obtained from the respective strains before the addition of L-arabinose (0 min). Mean values from at least three independent cultures are shown with the standard deviations.

lacZ fusion (*PgalM-lacZ*) on the chromosome (*PgalM-F*). *PgalM-F* and *ParaR-F* strains were cultured in A medium for 4 h; the mid-log-phase cells were then supplemented with L-arabinose to a final concentration of 2% (wt/vol) (Fig. 2B), and the levels of the reporter *lacZ* mRNA were determined by qRT-PCR. The expression level of *PgalM-lacZ* was increased in response to L-arabinose. This is consistent with the response of *galM* mRNA, as shown in our previous study (24). However, *ParaR-lacZ* expression was not affected by L-arabinose. Taken together, these results indicate that *araR* is transcribed from *PgalM* in L-arabinose-responsive manner as an operon and is also transcribed under the control of its own promoter constitutively.

Role of BS_B in expression of *araBDA* and *galM-araR* operons. Previously, we found the 16-bp consensus palindromic sequence ATGTTAGCGnTAcat based on the alignment of the three binding sites of *C. glutamicum* ATCC 31831 AraR (see Fig. S1A in the supplemental material). This motif is present between base pair positions +8 and +23 with respect to the *araB* transcription start site. To examine the role of BS_B on the expression of *araBDA*, we constructed a BS_B-deficient mutant strain, designated B-m,

FIG 3 Effects of BS_B mutation on the divergent *araBDA* and *galM-araR* operons. The relative mRNA levels of *araB*, *araD*, and *araA* (A) and *galM* and *araR* (B) were determined. The *C. glutamicum* ATCC 31831 WT strain and the AraR-binding site mutant B-m strains were grown in nutrient-rich A medium for 4 h, and the exponentially growing cells were then supplemented with L-arabinose to a final concentration of 2% (wt/vol). The mRNA levels in the cells incubated with sugars for 0, 5, and 15 min were determined by qRT-PCR and are presented relative to the value obtained from the wild-type cells before the addition of L-arabinose (0 min). The mean values from at least three independent cultures are shown with the standard deviations.

where CGA in the center of BS_B (ATGTTAGCGATAACAC) was substituted by ATC on the chromosome. We confirmed that this mutation leads to a drastic reduction of DNA-binding activity of AraR in previous *in vitro* experiments (24). B-m and the wild-type strain (*C. glutamicum* ATCC 31831) were cultured in A medium for 4 h, and the mid-log-phase cells were then supplemented with L-arabinose to a final concentration of 2%, as described earlier. qRT-PCR analyses revealed that, in the wild-type (WT) strain, the expression levels of the *araBDA* genes increased more than 20-fold within 5 min of L-arabinose supplementation and then decreased in the subsequent 10 min to some extent (Fig. 3A), as previously reported (24). In the absence of L-arabinose, the expression levels of *araBDA* in B-m were higher than those in the WT strain, and were comparable to the L-arabinose-induced levels in the WT strain. The effect of the BS_B mutation on the expression levels of *araBDA* is largely similar to that of deletion of the *araR* gene, as reported in our previous study (24), supporting that the mutation in the AraR binding site does not interfere with basal promoter activity and the stability of the mRNAs. These findings establish that BS_B is an essential *cis* element for the AraR-dependent regulation of the *araBDA* operon. However, the expression level of this operon in B-m decreased ~10-fold within 15 min of L-arabinose supplementation. The marked downregulation in response to L-arabinose has been also observed in an *araR* deletion mutant in our previous study (24), suggesting the involvement of an unknown L-arabinose-responsive regulatory mechanism.

BS_B overlaps the -10 region with respect to *galM*, which is divergently transcribed relative to *araB*. We examined the effects

FIG 4 *In vitro* binding of AraR to the *araE* promoter region. (A) The two AraR-binding sites in the *araE* upstream region (BS_{E1} and BS_{E2}). An inverted repeat sequence is indicated by arrows at the top of the alignment. Mutated nucleotides in BS_{E1} (E1-mut) and BS_{E2} (E2-mut) are shown below the respective original sequences. (B) EMSAs were performed using the AraR protein at different concentrations (lane 1, 0 nM; lane 2, 4 nM; lane 3, 8 nM; lane 4, 16 nM) and various DNA probes (WT, containing intact BS_{E1} and BS_{E2}; E1-mut, containing intact BS_{E2}; E2-mut, containing intact BS_{E1}; E1/2-mut, containing no intact AraR binding sites) at 1 nM. The free DNA and DNA-protein complex are indicated by white and black arrowheads, respectively. (C) Effects of L-arabinose on the binding of AraR to the intact or mutated *araE* promoter regions. EMSAs were carried out using the DNA probe at 1 nM, the AraR protein at 16 nM, and L-arabinose at 0, 1, 3, or 5 mM.

of BS_B mutation on the expression of the *galM-araR* operon by qRT-PCR as described above. In B-m, the levels of *galM* and *araR* transcripts were higher than those in the WT strain (Fig. 3B), although the effects of mutation of BS_B were smaller than those on the *araBDA* expression. This is consistent with the effects of *araR* deletion on these genes as shown in our previous study (24). Therefore, it is likely that the BS_B mutation has no effect on the basal promoter activity of the *galM* promoter, as in the case of the *araB* promoter described above. These findings indicate that binding of AraR to BS_B results in repression of both the divergently transcribed *araBDA* and *galM-araR* operons but to different degrees. L-Arabinose supplementation resulted in little changes in the expression levels of *galM* and *araR* in B-m, in contrast to the marked downregulation of *araBDA*, as described above.

***In vitro* binding of AraR to each of the two target sequences located in the *araE* promoter.** Two AraR-binding sites were previously detected upstream of *araE* by DNase I footprinting analysis (24). However, the AraR binding pattern of each site *in vitro* and the effects of the binding on the *araE* expression *in vivo* have not been investigated yet. The centers of BS_{E1} and BS_{E2} are separated by 53 bp which corresponds to 5.05 helix turns (10.5 bp per helix turn for B-DNA *in vitro* [28–30]). Thus, AraR may bind cooperatively to the two in-phase target sequences to form a loop structure upstream of *araE*. To assess the functionality of these two elements, we introduced substitution of CG by AT in the center of the palindromic sequence in both AraR binding sites (Fig. 4A). EMSA was performed using the *C. glutamicum* ATCC

FIG 5 Effects of 1 mM L-arabinose on the binding of AraR to three operators. EMSAs were carried out using a DNA probe at 1 nM and various concentrations of the AraR protein (0, 1, 2, 4, 8, or 16 nM) with or without 1 mM L-arabinose (Ara). The *araB* promoter fragment (*ParaB*), the *araE* promoter fragment (*ParaE*), and the *araE* promoter fragments with mutations in BS_{E2} (E2-mut) or BS_{E1} (E1-mut), the same as in Fig. 4, are used for DNA probes. The free DNA and DNA-protein complex are indicated by white and black arrowheads, respectively.

31831 AraR protein, which was expressed in *E. coli* and purified. The AraR protein reduced the electrophoretic mobility of the wild-type probe (WT; containing intact BS_{E1} and BS_{E2}) (Fig. 4B). Two shifted bands were detected depending on the amount of AraR. In contrast, one shifted band was observed when E1-mut (containing intact BS_{E2}) and E2-mut (containing intact BS_{E1}) were used as a DNA probe even in the presence of a high concentration of AraR. No shifted band was detected when E1/2-mut (containing no intact AraR binding sites) was used as a DNA probe. These results indicate that CG nucleotides in the center of palindrome of BS_{E1} and BS_{E2} are essential for binding of AraR under the conditions used. AraR appeared to have almost the same binding affinity for BS_{E1} and BS_{E2}, and the cooperative binding to these two sites was not clearly detected.

Next, we examined the effect of L-arabinose on the binding of AraR. EMSAs revealed that L-arabinose inhibited the AraR binding to the WT, E1-mut, and E2-mut probes similarly (Fig. 4C). Interestingly, the DNA binding affinity of the AraR protein for BS_{E1} and BS_{E2} was lower than that for BS_B in the presence of 1 mM L-arabinose, although almost the same affinity for these probes was observed in the absence of L-arabinose (Fig. 5; see Fig. S1 in the supplemental material). We also observed that a higher concentration of L-arabinose is required to inhibit the binding of AraR to BS_B compared to the binding to BS_{E1/2} (see Fig. S1E in the supplemental material). The difference in the binding affinities of AraR might result in different L-arabinose dose-response expressions of *araBDA* and *araE*. However, this hypothesis was not confirmed by the results of qRT-PCR under the conditions used (see Fig. S2 in the supplemental material).

Effects of BS_{E1} and/or BS_{E2} mutation on expression of *araE*.

To examine the role of the two AraR-binding sites upstream of *araE* on its expression *in vivo*, the aforementioned mutations of the AraR-binding sites were introduced into the *araE* promoter at the original locus in the WT strain, yielding E1-m, E2-m, and E1/2-m strains. For qRT-PCR analysis, the cells were grown in nutrient-rich A medium, and then the exponentially growing cells were supplemented with 2% (wt/vol) L-arabinose, as described

earlier. In the absence of L-arabinose, the level of *araE* mRNA in E2-m was comparable to that in the WT strain (Fig. 6). In E1-m, the expression level of *araE* was ~5-fold higher than that in the WT strain but was significantly lower than the L-arabinose-induced level in the WT strain. In E1/2-m, the *araE* expression level was ~30-fold higher than that in the WT strain in the absence of L-arabinose. The effect of the BS_{E1/E2} mutation was comparable to that of *araR* deletion, as reported in our previous study (24), supporting that the mutation in the AraR binding sites do not interfere with basal activity of the *araE* promoter. These results indicate that the two AraR binding sites exert repression of *araE* independently, but the binding to BS_{E1} leads to stronger repression than the binding to BS_{E2}. The levels of *araE* mRNA in the WT and E2-m strains increased ~15-fold within 5 min of L-arabinose supple-

FIG 6 Expression of *araE* in AraR-binding site mutants. Single mutations in BS_{E1} (E1-m) and BS_{E2} (E2-m) and a double mutation in these sites (E1/2-m) were introduced into the *araE* promoter region in the *C. glutamicum* ATCC 31831 chromosome. The three mutants and the wild-type (WT) strain were grown in nutrient-rich A medium for 4 h, and the exponentially growing cells were then supplemented with L-arabinose at a final concentration of 2% (wt/vol). The levels of *araE* mRNA in the cells incubated with L-arabinose for 0, 5, and 15 min were determined by qRT-PCR. The mRNA levels are presented relative to the value obtained from the WT cells before supplementation with L-arabinose (0 min). Mean values from at least three independent cultures are shown with the standard deviations.

FIG 7 Effects of disruption of *araA* on the L-arabinose-inducible gene expression. The *C. glutamicum* ATCC 31831 wild type (WT) and the mutants deficient in the AraR-binding site upstream of *araB* in the background of the wild-type strain (WT/B-m) or the *araA* deletion mutant ($\Delta araA/B$ -m). These strains were grown in nutrient-rich A medium for 4 h, and the exponentially growing cells were then supplemented with L-arabinose to a final concentration of 2% (wt/vol). The levels of *araB* and *galM* (A) and *araE* (B) mRNAs in the cells incubated with L-arabinose for 0 and 15 min were determined by qRT-PCR. The mRNA levels are presented relative to the value obtained from the B-m cells before supplementation with L-arabinose (0 min). Mean values from at least three independent cultures are shown with the standard deviations.

mentation, while *araE* in E1-m was upregulated ~3-fold. The induced level of *araE* mRNA in E1-m was the same as that in the WT strain. In E1/2-m, the expression of *araE* remained high at least for 15 min in the presence of L-arabinose. After the induction, the expression levels of *araE* in the WT, E1-m, and E2-m strains slightly decreased in the subsequent 10 min in contrast to that in E1/2-m. The slight reduction might reflect the AraR-dependent regulation in response to the reduction of an intracellular level of L-arabinose by its catabolism. In all three mutant strains, the other L-arabinose utilization genes were upregulated in response to L-arabinose, as observed in the WT strain.

Effects of L-arabinose catabolism on expression of the L-arabinose utilization genes. Our previous study revealed that catabolism of L-arabinose taken up by cells is not required for derepression of the AraR-controlled genes (24). However, the AraR-independent repression in response to L-arabinose, as shown in Fig. 3, may be dependent on catabolism of the substrate. To examine this hypothesis, we introduced the aforementioned BS_B mutation into the chromosome of an *araA* deletion mutant, yielding the $\Delta araA/B$ -m strain (Fig. 7). The $\Delta araA$ strain cannot grow on L-arabinose as the sole carbon source (24). For qRT-PCR, the cells were grown in A medium, and then the exponentially growing cells were supplemented with 2% (wt/vol) L-arabinose, as

described earlier. In the absence of L-arabinose, *araB* was highly expressed in $\Delta araA/B$ -m, as well as in B-m, as described in Fig. 3. The level of *araB* mRNA in $\Delta araA/B$ -m was unchanged within 15 min of L-arabinose supplementation in contrast to the marked downregulation in B-m. These results indicate that catabolism of L-arabinose is responsible for the strong repression of *araB* expression in an AraR-independent manner. Similar results were observed for *galM*, although the difference in the expression level between B-m and $\Delta araA/B$ -m was smaller than that in the case of *araB* in the presence of L-arabinose.

We also found that the L-arabinose-responsive upregulation of *araE* was impaired by the mutation of BS_B (Fig. 7B). This is probably due to enhanced catabolism of L-arabinose by constitutive upregulation of the *araBDA* operon. In fact, the upregulation of *araE* in response to L-arabinose was restored by deletion of *araA* as observed in $\Delta araA/B$ -m.

DISCUSSION

In *C. glutamicum* ATCC 31831, L-arabinose catabolic genes (*araBDA*), as well as its uptake gene (*araE*), are strongly inducible in response to L-arabinose, in contrast to their regulator gene (*araR*) that is also upregulated but to a much smaller extent (24). The LacI family transcriptional regulator AraR acts as a repressor of these genes, which are derepressed by the inducer L-arabinose (24). In the present study, we elucidated the *in vivo* roles of three binding sites of AraR in the regulation of its target genes clustered on the chromosome, as described below.

Two AraR binding sites upstream of *araE* (BS_{E1} and BS_{E2}) were shown to be the functional *cis* elements controlling the L-arabinose uptake gene (Fig. 6). BS_{E1} is sufficient for the strong repression in the absence of L-arabinose, as observed in the BS_{E2} mutant, although mutation of both sites are required for full derepression. Thus, BS_{E1} plays a pivotal role in the AraR-dependent regulation of *araE*. EMSAs indicated that the binding affinity of the AraR protein for the primary site, BS_{E1}, is almost the same as that for the additional site, BS_{E2} (see Fig. S1C and D in the supplemental material). The binding activity of AraR for the *araE* promoter region was similarly reduced by L-arabinose irrespective of mutation of BS_{E1} or BS_{E2}. Therefore, it is likely that the weaker repression of *araE* exerted by the binding of AraR to BS_{E2}, as observed in the BS_{E1} mutant (Fig. 6), is ascribed to its location in the promoter but not to its binding affinity. BS_{E2} overlaps the -35 region of *araE*, whereas BS_{E1} is located immediately downstream of its transcriptional start site. The binding of AraR to the latter primary site probably prevents the initiation or elongation of transcription by RNA polymerase more effectively than the former additional site. The independent binding to two sites with the same affinity, but the different effects on the repression activity might confer flexibility in the AraR-dependent regulation of the L-arabinose uptake gene by competitive binding to these sites under some conditions. This is in contrast to the tight regulation exerted by the cooperative binding to multiple sites, as reported for well-studied members of the LacI/GalR family (31, 32) and other types of L-arabinose-responsive transcriptional regulators, i.e., *B. subtilis* AraR and *E. coli* AraC. In *B. subtilis*, DNA looping by cooperative binding of the GntR family regulator AraR plays an important role in the strong repression of the L-arabinose uptake and catabolic genes in the absence of the substrate (18, 19). *E. coli* AraC belonging to the AraC/XylS family represses the transcription of the L-arabinose catabolic operon via a DNA-looping mechanism in the

absence of L-arabinose, and its binding mode is changed by L-arabinose to activate the same operon (15).

We showed here that mutation of the single AraR-binding site (BS_B) within the intergenic region between *araB* and *galM*, the genes which are located in the arabinose cluster in *C. glutamicum* ATCC 31831 (Fig. 1), resulted in increased expression of both the divergently transcribed *araBDA* and *galM-araR* operons in the absence of L-arabinose (Fig. 3). The expression levels of these operons in the BS_B mutant were comparable to the respective L-arabinose-induced levels in the WT strain. It should be noted that no additional AraR binding sites were detected in the proximity of BS_B , unlike in the promoter region of *araE*. It is also unlikely that BS_{E1} and BS_{E2} are directly involved in the regulation of *araBDA* and *galM-araR* because the two AraR-binding sites are located far (>2 kb) from the promoter regions of these operons. We confirmed that mutation of either BS_{E1} or BS_{E2} has little effect on the expression levels of *araBDA* and *galM-araR* (data not shown). Taken together, BS_B is a sole essential *cis* element for the L-arabinose-responsive AraR-mediated regulation of its downstream operons transcribed in the opposite directions. However, the effect of BS_B mutation on *araBDA* expression was much larger than that on *galM-araR* expression, indicating that the binding of AraR to this site is more effective in repression of this L-arabinose catabolic operon. It is noteworthy that BS_B is located between bp +8 and +23 with respect to the transcriptional start site of *araB* similarly to the primary AraR binding site (BS_{E1}) for the strong repression of *araE*, but it overlaps the -10 region of the divergent *galM* promoter. These findings seem to be consistent with the role of the AraR-binding site location in the degree of repression, as described above for *araE*, although differences in full activity among these promoters probably affect the degree of repression.

Based on the findings described above, we conclude that AraR strongly represses the expression of the L-arabinose catabolic genes (*araBDA*) and its uptake gene (*araE*) through binding to the 16-bp consensus palindromic sequence located immediately downstream of each of their transcriptional start sites. The affinity of the binding of AraR to the same locations in the two promoter regions was shown to be the same in the absence of L-arabinose by our EMSAs (see Fig. S1 in the supplemental material). However, we observed higher affinity for BS_B than for BS_{E1} and BS_{E2} in the presence of L-arabinose (Fig. 5), implying that the required concentration of L-arabinose for derepression of *araE* is lower than *araB*. The different characteristics between L-arabinose-responsive promoters may underlie an optimal coordination of uptake and catabolism of the sugar substrate, in combination with the additional AraR binding site that exists upstream of *araE* but not of *araBDA* and *galM-araR*. However, we could not verify the hypothesis by analyses of the induction of *araB* and *araE* in response to various concentrations of L-arabinose under the conditions used (see Fig. S2 in the supplemental material). The different characteristics between L-arabinose-responsive promoters may underlie an optimal coordination of uptake and catabolism of the sugar substrate, in combination with the additional AraR binding site that exists upstream of *araE* but not of *araBDA* and *galM-araR*. It should be noted that an intracellular level of the inducer L-arabinose is up- or down-regulated through its uptake and catabolism, respectively, under the direct control of AraR, creating a complicated feedback loop, as proposed for the AraC-dependent regulatory system in *E. coli* (33–35). In this regulatory network, the expression of the regulator gene (*araR*) should have an important role in the dynamic response to the changes in the substrate availability. In this context, our results

indicate that AraR autoregulates its own expression by acting as a transcriptional repressor of the *galM-araR* operon through the binding to BS_B in the *galM* promoter (Fig. 1B and 3B). It is conceivable that the negative autoregulation maintains the expression level of the regulator in the absence of L-arabinose, but its derepression in the presence of L-arabinose contributes to a quick response to changes in the sugar substrate availability. In addition to the L-arabinose-responsive transcription, it is noteworthy that *araR* is constitutively transcribed from its own promoter whose activity was not induced by supplementation of L-arabinose (Fig. 2B), which is consistent with no binding of AraR to the *araR* promoter region, as shown in our previous study (24). The constitutive promoter may support the basal level of the regulator, although minimal difference in the response to L-arabinose between the expression levels of *galM* and *araR* was observed under the conditions used (Fig. 3). A similar regulation of a LacI/GalR family regulator, namely, transcription from two independent promoters, an autoregulated promoter and another constitutive one, has also been reported in *Streptomyces griseus* CebR, a regulator of cellulose/cello-oligosaccharide catabolism (36).

In addition to the AraR-dependent regulation, we detected AraR-independent regulation of L-arabinose utilization genes. Due to the mutation of BS_B , the expression of the L-arabinose catabolic operon (*araBDA*) was derepressed in the absence of L-arabinose, but the expression level rapidly decreased after supplementation with L-arabinose (Fig. 3). A similar strong L-arabinose-dependent repression has been observed in an *araR* deletion mutant previously (24). On the other hand, by deletion of an L-arabinose catabolic gene (*araA*) in the background of these mutant strains, the high expression level of the *araBDA* operon remained in the presence of L-arabinose (Fig. 7A), indicating that the L-arabinose-dependent and AraR-independent repression requires L-arabinose catabolism. The repression of the L-arabinose utilization genes through catabolism of the substrate sugar also has been observed in *E. coli* (37). In the case of *E. coli*, not only blocking L-arabinose metabolism but also adding exogenous cyclic AMP (cAMP) countermand the downregulation of L-arabinose metabolic genes, which appears to be a result of carbon catabolite repression (38). This catabolite repression might avoid excessive expenditure of energy for overproduction of enzymes through the induction process. The global carbon catabolite repression system, mediated by cAMP in *E. coli*, has never been found in *C. glutamicum*. Moreover, it should be noted that *C. glutamicum* has the ability to consume various carbon sources, including a mixture of D-glucose and L-arabinose, simultaneously (21). Thus, an unidentified unique regulatory system may be involved in the AraR-independent repression of the arabinose catabolic operon in *C. glutamicum* ATCC 31831. The L-arabinose uptake gene (*araE*) is also markedly downregulated in response to L-arabinose in the *araR* deletion mutant (24) but not clearly in the mutant deficient in the two AraR-binding sites upstream of *araE* (Fig. 6), implying the same repression mechanism dependent on L-arabinose catabolism as in the case of the catabolic operon (*araBDA*). This mechanism may explain the current observation that the L-arabinose-responsive upregulation of *araE* was impaired by the mutation of BS_B indirectly through the upregulation of the *araBDA* operon (Fig. 7B). However, in this case, it is also plausible that downregulation of an intracellular level of L-arabinose by the enhancement of its catabolism suppresses the AraR-mediated regulation of *araE*. In addition to the present findings regarding the AraR-dependent regulatory mechanism, further study of the

AraR-independent mechanism will represent a major advance in understanding the complex regulatory network for optimal metabolism of the pentose sugar L-arabinose, which is important for the development of bioprocesses for efficient utilization of renewable lignocellulosic feedstocks, in the biotechnologically important *C. glutamicum*.

ACKNOWLEDGMENT

This work was financially supported in part by the New Energy and Industrial Technology Development Organization (NEDO), Japan.

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