JB
Journals.ASM.org

The LacI-Type Transcriptional Regulator AraR Acts as an L-Arabinose-Responsive Repressor of L-Arabinose Utilization Genes in *Corynebacterium glutamicum* ATCC 31831

Takayuki Kuge,[a,b] Haruhiko Teramoto,[a] Hideaki Yukawa,[a] Masayuki Inui[a,b]

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

The *Corynebacterium glutamicum* ATCC 31831 *araBDA* operon consists of three L-arabinose catabolic genes, upstream of which the *galM*, *araR*, and *araE* genes are located in opposite orientation. *araR* encodes a LacI-type transcriptional regulator that negatively regulates the L-arabinose-inducible expression of *araBDA* and *araE* (encoding an L-arabinose transporter), through a mechanism that has yet to be identified. Here we show that the AraR protein binds *in vitro* to three sites: one upstream of *araBDA* and two upstream of *araE*. We verify that a 16-bp consensus palindromic sequence is essential for binding of AraR, using a series of mutations introduced upstream of *araB* in electrophoretic mobility shift assays. Moreover, the DNA-binding activity of AraR is reduced by L-arabinose. We employ quantitative reverse transcription-PCR (qRT-PCR) analyses using various mutant strains deficient in L-arabinose utilization genes to demonstrate that the prominent upregulation of *araBDA* and *araE* within 5 min of L-arabinose supplementation is dependent on the uptake but independent of the catabolism of L-arabinose. Similar expression patterns, together with the upregulation by *araR* disruption without L-arabinose, are evident with the apparent *galM-araR* operon, although attendant changes in expression levels are much smaller than those realized with the expression of *araBDA* and *araE*. The AraR-binding site upstream of *araB* overlaps the −10 region of the divergent *galM* promoter. These observations indicate that AraR acts as a transcriptional repressor of *araBDA*, *araE*, and *galM-araR* and that L-arabinose acts as an intracellular negative effector of the AraR-dependent regulation.

Corynebacterium glutamicum is a Gram-positive *Actinobacteria* species with a high G+C content in its genomic DNA. It is currently used in large-scale industrial production of amino acids such as L-glutamate and L-lysine (1, 2). Moreover, investigation into its application in the production of fuels and commodity chemicals has started to bear fruit (3–5). This is predicated on its potential to utilize cellulosic biomass, a renewable energy source that should play an important role in the global renewable energy of the future (6, 7). Cellulosic biomass contains significant amounts of pentose sugars, such as D-xylose and L-arabinose, that are often difficult for conventional bioprocesses to exploit. Recent advances in metabolic engineering of *C. glutamicum* have helped to open up new possibilities for efficient utilization of substrates containing mixtures of D-glucose, D-xylose, and L-arabinose (8–11). An understanding of the transcriptional regulation of sugar metabolism genes in *C. glutamicum* is an attractive avenue toward optimization of utilization of such sugar mixtures.

L-Arabinose is utilized by a Gram-negative bacterium, *Escherichia coli*, and a low-G+C Gram-positive bacterium, *Bacillus subtilis*, via a common pathway. L-Arabinose taken up by cells is sequentially converted to L-ribulose, L-ribulose 5-phosphate, and D-xylulose 5-phosphate by the actions of L-arabinose isomerase (encoded by *araA*), L-ribulokinase (encoded by *araB*), and L-ribulose 5-phosphate 4-epimerase (encoded by *araD*), respectively. D-Xylulose 5-phosphate is further catabolized through the pentose phosphate pathway. Despite the conservation of this pathway among various bacteria, two distinct regulatory systems of the L-arabinose utilization genes have been well established so far. In *E. coli*, AraC acts as an activator and as a repressor that regulates transcription of the genes required for the catabolism (*araBAD*) and uptake (*araE* and *araFGH*) of L-arabinose (12). AraC is composed of an N-terminal arabinose-binding and dimerization domain and a C-terminal DNA-binding domain. The expression of the *araBAD* operon and the AraC-encoding gene (*araC*), which are divergently transcribed, is repressed by binding of AraC to the promoter region forming a DNA loop in the absence of L-arabinose (13–15). In the presence of L-arabinose, three promoters, P*araBAD*, P*araE*, and P*araFGH*, are activated, and P*araC* is derepressed (16). In *B. subtilis*, AraR, which is structurally different from *E. coli* AraC, controls the transcription of several genes involved in the uptake and degradation of arabinose-containing polysaccharides, in addition to the genes corresponding to the *E. coli* AraC regulon (17–19). AraR comprises an N-terminal DNA-binding domain homologous to the GntR family proteins and a C-terminal effector-binding domain that shows similarity to members of the LacI/GalR family (20, 21). *B. subtilis* AraR binds to five different promoter regions with distinct affinities (P*araABDLMNPQ-abfA*, P*araE*, P*abnA*, P*xsa*, and P*araR*) (18, 19, 22). Moreover, an L-arabinose transporter encoded by *araE* under the control of AraR is also responsible for the uptake of D-xylose and D-galactose (23). Therefore, AraR is thought to play a central role in the regulation of utilization of the multiple sugars in *B. subtilis*. It is noted that the L-arabinose catabolic and uptake genes are also

Received 12 March 2014 **Accepted** 2 April 2014
Published ahead of print 4 April 2014
Address correspondence to Masayuki Inui, mmg-lab@rite.or.jp.

doi:10.1128/JB.01655-14

subject to carbon catabolite repression, controlled by the cyclic AMP (cAMP)-dependent transcriptional regulator CRP in *E. coli* (24) and the LacI family transcriptional regulator CcpA in *B. subtilis* (25). These distinct regulatory systems are responsible for inhibition of L-arabinose utilization in the presence of D-glucose.

Most strains of *C. glutamicum* tested cannot grow using L-arabinose as the sole carbon source owing to the lack of the pentose sugar utilization pathway (26). However, *C. glutamicum* ATCC 31831 was recently shown to efficiently utilize L-arabinose due to the presence of *araBDA* and *araE* in its genome (26). The expression of these genes associated with L-arabinose utilization is induced in the presence of L-arabinose and is also upregulated by inactivation of *araR*, encoding a LacI family transcriptional regulator, in the absence of L-arabinose (26). Although the AraR protein is suggested to be involved in the regulation of the L-arabinose-inducible genes in this strain, whether or not AraR directly regulates the relevant genes remains to be verified. It should be noted that AraR of *C. glutamicum* ATCC 31831 and AraR of *B. subtilis* belong to distinct families, although the latter protein reveals a mosaic structure with a C-terminal effector-binding domain that has some similarity to the former, LacI family protein. Furthermore, *C. glutamicum* ATCC 31831 simultaneously utilizes L-arabinose and D-glucose (26), apparently in contrast to the glucose repression of L-arabinose utilization genes in *E. coli* and *B. subtilis* described earlier. Thus, elucidation of the regulatory mechanism of the L-arabinose utilization genes in *C. glutamicum* ATCC 31831 should extend our understanding of the regulatory systems of sugar metabolism diversified among bacterial species.

In this study, we show that *C. glutamicum* ATCC 31831 AraR binds *in vitro* to 16-bp consensus palindromic sequences found separately at one site upstream of *araBDA* and at two sites upstream of *araE*. The AraR-binding site upstream of *araB* overlaps the promoter of the apparent *galM-araR* operon, divergently transcribed from *araBDA*. The binding activity of AraR is reduced by L-arabinose. In addition, we show effects of *araR*, *araB*, *araD*, *araA*, or *araE* deletion on L-arabinose-inducible gene expression *in vivo* that suggest that AraR acts as a transcriptional repressor of *araBDA*, *araE*, and *galM-araR*. The AraR-mediated expression of these multiple transcriptional units is coordinately derepressed in the presence of L-arabinose in response to uptake of the sugar, but independent of its catabolism.

MATERIALS AND METHODS

Bacterial strains. *C. glutamicum* ATCC 31831 was used as a wild-type strain in this study. The *araB*, *araD*, and *araR* single-deletion mutant strains were described previously (26). *E. coli* strains JM109 (TaKaRa), JM110 (27), and BL21(DE3) (28) were used for cloning and/or expression of genes of interest.

Culture conditions. For genetic manipulations, *E. coli* strains were grown at 37°C in Luria-Bertani (LB) medium (27). *C. glutamicum* strains were grown at 33°C in nutrient-rich A medium [2 g/liter yeast extract, 7 g/liter Casamino Acids, 2 g/liter $(NH_2)_2CO$, 7 g/liter $(NH_4)_2SO_4$, 0.5 g/liter KH_2PO_4, 0.5 g/liter K_2HPO_4, 0.5 g/liter $MgSO_4 \cdot 7H_2O$, 6 mg/liter $FeSO_4 \cdot 7H_2O$, 4.2 mg/liter $MnSO_4 \cdot H_2O$, 0.2 mg/liter biotin, 0.2 mg/liter thiamine] (29) with 4% glucose. Where appropriate, the culture medium was supplemented with 50 μg ml^{-1} of kanamycin.

For analytical purposes, a *C. glutamicum* starter culture was grown aerobically in 10 ml of nutrient-rich A medium in a 100-ml test tube overnight. The cells were inoculated into fresh medium at a dilution of 100-fold or higher. 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 and/or D-glucose at the stated concentrations.

DNA technique. Chromosomal DNA was isolated from *C. glutamicum* cells by using a GenomicPrep cell and tissue DNA isolation kit (GE Healthcare, Buckinghamshire, England) according to the manufacturer's instructions, but modified by using 10 mg ml^{-1} lysozyme at 37°C for 1 h. Plasmid DNA was isolated using a QIAprep Spin miniprep kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, but modified for extraction from *C. glutamicum* cells by using 10 mg ml^{-1} lysozyme at 37°C for 1 h.

PCR was performed using a model 480 DNA thermal cycler (Perkin-Elmer, MA). After the reaction mixture was heated at 96°C for 2 min, the PCR proceeded with 30 cycles of 15 s at 96°C, 30 s at 55°C, and 2 min at 68°C by using DNA polymerase KOD Plus (Toyobo, Osaka, Japan).

C. glutamicum cells were transformed by electroporation as described previously (30). *E. coli* cells were transformed by the $CaCl_2$ procedure (27).

DNA sequencing was performed by the dideoxy chain termination method (31), using an ABI Prism 3100xl genetic analyzer (Applied Biosystems, Foster City, CA) and a BigDye Terminator, version 3.1, cycle sequencing kit (Applied Biosystems). DNA sequence data were analyzed with the Genetyx program (Genetyx Corporation, Tokyo, Japan). Sequences were aligned by use of the ATGC program (Genetyx Corporation).

Mutant construction. The upstream and downstream regions of the target gene for deletion were amplified using sets of primers summarized in Table 1. The resultant amplicons were fused and cloned into pCRD206 (32), a suicide vector for markerless gene disruption. The resultant plasmids, pCRF500 and pCRF501, were used for in-frame deletion of *araA* and *araE*, respectively. *C. glutamicum* was subsequently transformed with the respective plasmid DNA, and screening for deletion mutants was performed as described previously (32). Deletion of the target genes was confirmed by PCR.

Quantitative reverse transcription-PCR (qRT-PCR) analysis and rapid amplification of cDNA ends (RACE). Total RNA was prepared from *C. glutamicum* cells by using an RNeasy minikit (Qiagen) according to the manufacturer's instructions. Single-stranded cDNA was synthesized from 0.2 μg of total RNA by using PrimeScript reverse transcriptase (TaKaRa, Osaka, Japan) with a random hexamer mixture as a primer in 20 μl of reaction mixture, and then 2 μl of the cDNA mixture was added as a template to 18 μl of reaction mixture containing each primer (0.3 μM) and Power SYBR green PCR master mix (Applied Biosystems). After the reaction mixture was heated at 95°C for 10 min, PCRs proceeded via 40 cycles of 15 s at 95°C and 40 s at 60°C. The amount of amplified DNA was monitored by fluorescence at the end of each cycle by using an Applied Biosystems 7500 Fast real-time PCR system. The primers used are listed in Table 1. The relative abundances of the target mRNAs were quantified based on the cycle threshold value. To standardize the results, the relative abundance of 16S rRNA was used as the internal standard.

The 5′ end of mRNA was determined by RACE, using the primers summarized in Table 1. Using a 5′-Full RACE core set (TaKaRa, Osaka, Japan), single-stranded cDNA synthesized from total RNA by using the 5′-phosphorylated primer was self-ligated with T4 RNA ligase. The first PCR proceeded using inverted primers, and then the second PCR proceeded using nested inverted primers. The amplified DNA was inserted into pUC118 HincII/BAP by using a Mighty Cloning Reagent set (TaKaRa, Osaka, Japan). More than 10 clones from *E. coli* transformed with the resulting plasmid were sequenced.

Purification of the AraR protein expressed in *E. coli*. A DNA fragment containing the *araR* gene was amplified by PCR using primers AraR-Ex-F and AraR-Ex-R (Table 1). The amplified DNA was digested with NdeI and EcoRI and was inserted into the corresponding site of the pET-28a expression vector (Merck KGaA, Darmstadt, Germany). The resulting plasmid, pCRF502, contains the *araR* gene fused to a His tag sequence at the N terminus. *E. coli* BL21(DE3) cells transformed with pCRF502 were

TABLE 1 Primers used in this study

Primer purpose and name	Sequence (5′–3′)	Overhanging restriction site[a]
Plasmid construction for gene deletion		
araA-ML-U-F	GCTCTAGAGTGCTGACGATGATGGCGGA	XbaI
araA-ML-U-R	CTCTACTAGTTCCATGTCGATCGAAGACCA	SpeI
araA-ML-D-F	GCTCTAGAAGTGCTATGGCAGTGCAACT	XbaI
araA-ML-D-R	CTCTACTAGTACTCACTGCGAACAAGGTCA	SpeI
araE-ML-U-F	GCTCTAGATCGCTCGGACACCGTCACAT	XbaI
araE-ML-U-R	GCGGATCCACCGAAGAAGTAGATGTACG	BamHI
araE-ML-D-F	CGGGATCCATCCCGCTCATCGTCGAGAA	BamHI
araE-ML-D-R	GCGATATCTGAGAGATGCCGTTGGTGCT	EcoRV
Plasmid construction for gene expression		
AraR-Ex-F	GGAATTCCATATGAGCTCCACCCAG	NdeI
AraR-Ex-R	GGAATTCTCAGAGCTGTGTGAAATC	EcoRI
RT-PCR analysis		
16S-F	CAGGTCTCTGGGCAGTAACTGA	
16S-R	CGTTTACGGCATGGACTACCA	
araA-F	GCGCCAATGACAACGTCAT	
araA-R	TCATTAGCCTGGGTGTGAAGGT	
araB-F	GGATCGAGCTCTTCAGTGAGGTT	
araB-R	CACCGGAGACGAAGTTGTAGGT	
araD-F	GCGATGGTGGTGTGCAACA	
araD-R	GGTCGAATGAGTGTGCACGAT	
araE-F	CATGTGGCCGATCATCCA	
araE-R	CGGCGATGACCATGAACA	
araR-F	GCACGCTGTCAAGCATTGA	
araR-R	GGTCTCTTCGGCATTTTTCATC	
galM-F	GGTCTTCGCCACCTTCTCTCT	
galM-R	GTGGTGTCGCCGACATTGT	
5′-RACE		
araB-RA-RT[b]	TGAGAACTAGGTCG	
araB-RA-F1	ACGGAGTTGAAAGTATGGAG	
araB-RA-R1	GTTCGGTTCCTTGATGAC	
araB-RA-F2	AATTCGGATCGACCCGCATT	
araB-RA-R2	TTTGAGAGCGGAGCTTCGTA	
araE-RA-RT[b]	GATCATCGTCTGGT	
araE-RA-F1	CGACCGTACATCTACTTCTT	
araE-RA-R1	GTTGAACAGTCTCTGTCATC	
araE-RA-F2	TCCTCTTCTCTGCGCTGGTA	
araE-RA-R2	GCTGTCTGAGCGGATGATGT	
galM-RA-RT[b]	TGCCATCGGTGTAGA	
galM-RA-F1	TCCACGGTGGTGTTCTCGTT	
galM-RA-R1	TTGATCGTGACCTGTTTTCC	
galM-RA-F2	CCGACGGCATCTACACCGAT	
galM-RA-R2	CTTCTGGGGCGATGTGTCAT	
Electrophoretic mobility shift assay		
PA-F	TGGTGCTGCCATCGGTGTAG	
PA-R	AGCTTCGTATTGGTGTTATCGCT	
PB-F	CCTCGAGTGAGTAGCTCCAG	
PB-R	CTTCTGGGGCGATGTGTCAT	
PC-F	TCTGGGTTCTCGGGTGATAA	
PC-R	GCTGTCTGAGCGGATGATGT	
PD-F	ACCGTCTCCTCCCGATCAGA	
PD-R	TGGGTGGAGCTCATGGTGAT	
P2-F	TGCGATCCGGTCACT	
P3-F	AATGCGGGTCGATCCGAA	
P4-F	ACGGTCCACGACACT	
P5-F	GTTCGGTTCCTTGATGAC	
P6-R	GTGTGATGCTTGCTTGAC	
P7-R	GATGGCCGTTAAGAAC	
P8-R	CTCAAATGTGATCCGAAGC	
P9-R	ACGGAGTTGAAAGTATGGAG	
DNase I footprinting analysis		
pUC-IR-Fw	TTGTAAAACGACGGCCAGTG	
pUC-IR-Rv[c]	GGAAACAGCTATGACCATGA	
araB-DF-F	CTTCTGGGGCGATGTGTCAT	
araB-DF-R	AGCTTCGTATTGGTGTTATCGCT	
araE-DF-F	CGAGCGACAAACACGACA	
araE-DF-R	TTGTCCTGGTTCTTCGAC	

[a] The restriction site overhangs used in the cloning procedure are underlined in the sequences.
[b] 5′-Phosphorylated primer.
[c] 5′-IRD700-labeled primer.

grown at 37°C in 100 ml of LB medium supplemented with kanamycin (50 μg ml^{-1}). The recombinant gene was expressed in exponentially growing cells (optical density at 610 nm [OD_{610}] = 0.6) by adding 1 mM isopropyl-β-D-thiogalactopyranoside. After 1 h of incubation, the cells were harvested by centrifugation. The His-tagged AraR protein was extracted and purified by affinity column chromatography, using a Ni-nitrilotriacetic acid (Ni-NTA) Fast Start kit (Qiagen). The AraR protein was loaded onto a gel filtration column (PD-10 column; GE Healthcare UK Ltd., Little Chalfont, United Kingdom) equilibrated with buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol and eluted with the same buffer. The resultant AraR protein was used for an electrophoretic mobility shift assay (EMSA).

EMSA. The purified His-tagged AraR protein at the indicated concentrations was incubated with a DNA probe in 20 μl of binding buffer containing 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 2.5 mM $MgCl_2$, 0.45 mM EDTA, 0.5 mM dithiothreitol (DTT), 0.05% (vol/vol) Nonidet P-40, and 10% (vol/vol) glycerol for 25 min at 25°C. The binding reaction mixture was subjected to electrophoresis on a 6% polyacrylamide gel containing 5% (vol/vol) glycerol in 0.5× Tris-borate-EDTA (TBE) electrophoresis buffer, and the DNA probe was detected with SYBR green. DNA probes were prepared by PCRs using plasmids containing the promoter regions of interest as templates. Primers used are summarized in Table 1.

DNase I footprinting analysis. A labeled DNA fragment was prepared by PCR, using pUC-IR-Fw and a 5′-IRD700-labeled primer, pUC-IR-Rv. pUC118 (TaKaRa) containing the region between positions +90 and −34 with respect to the transcription start site of *araB* or the region between positions +85 and −104 with respect to the transcription start site of *araE* was used as the template. The purified AraR protein at the stated concentrations was incubated with a DNA probe in 20 μl of binding buffer containing 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 2.5 mM $MgCl_2$, 0.45 mM EDTA, 0.5 mM dithiothreitol, 0.05% Nonidet P-40, and 10% glycerol for 25 min at room temperature. Four microliters of binding buffer containing 1 to 2 mU of DNase I (TaKaRa), 5 mM $MgCl_2$, and 10 mM $CaCl_2$ was then added and incubated for 1 min, followed by the addition of 2 μl of 325 mM EDTA and subsequent heating at 80°C for 10 min. The samples were mixed with IR2 stop solution (Li-Cor, Lincoln, NE), heated at 95°C for 3 min, and separated in a 5.5% KB Plus gel matrix (Li-Cor), using a Li-Cor 4300 DNA analyzer. DNA sequencing reaction mixtures using the same IRD700-labeled primer and a DYEnamic direct cycle sequencing kit with 7-deaza-dGTP (GE Healthcare UK Ltd.) were subjected to the same gel analysis.

RESULTS

L-Arabinose-inducible expression of the L-arabinose utilization cluster genes. On the chromosome of *C. glutamicum* ATCC 31831, six genes, i.e., *araA*, *araB*, *araD*, *araE*, *galM*, and *araR*, are located in a cluster (Fig. 1) (26). *araA*, *araB*, and *araD*, each of which is essential for L-arabinose utilization in this strain (26), encode arabinose isomerase, ribulokinase, and ribulose-5-phosphate 4-epimerase, respectively. An H^+ symporter encoded by *araE* is involved in L-arabinose uptake (8). *galM* and *araR* encode a putative aldose 1-epimerase and a LacI-type transcriptional regulator, respectively. The *araB* gene is oriented in the same direction as its two downstream genes, *araD* and *araA*. The *araB-araD* and *araD-araA* intergenic regions are 13 and 35 bp long, respectively. Upstream of the *araB* gene, *galM* is divergently oriented. *araR* and *araE* are located downstream of *galM*, in the same direction. The *galM-araR* and *araR-araE* intergenic regions are 26 and 234 bp long, respectively.

We previously reported that the expression levels of *araBDA* and *araE* in this L-arabinose utilization gene cluster are dramatically increased in exponentially growing cells in minimal medium supplemented with L-arabinose compared with that in cells grown on D-glucose (26). Here we examined changes in the expression of

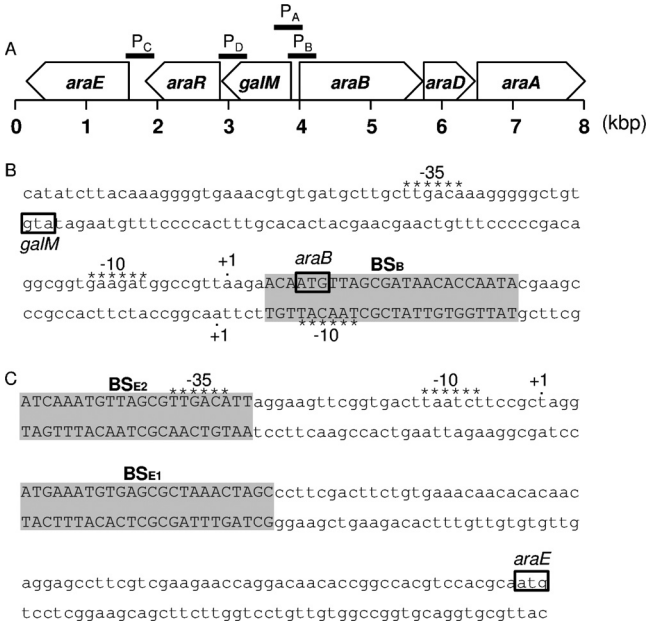

FIG 1 Cluster of L-arabinose utilization genes on the chromosome of *C. glutamicum* ATCC 31831. (A) The locations and directions of transcription of the six genes (*araA*, *araB*, *araD*, *araE*, *araR*, and *galM*), based on a previous study (26), are shown, and the fragments used as DNA probes (P_A, P_B, P_C, and P_D) in electrophoretic mobility shift assays are indicated by solid lines. The nucleotide sequences of the *araB-galM* intergenic region (B) and the *araE* upstream region (C) are shown. The translation start codons are boxed, and the putative −10 and −35 regions, based on the transcription start sites (+1) determined, are indicated by asterisks above the sequences. The regions protected by AraR in DNase I footprinting analysis are indicated in capital letters against a gray background.

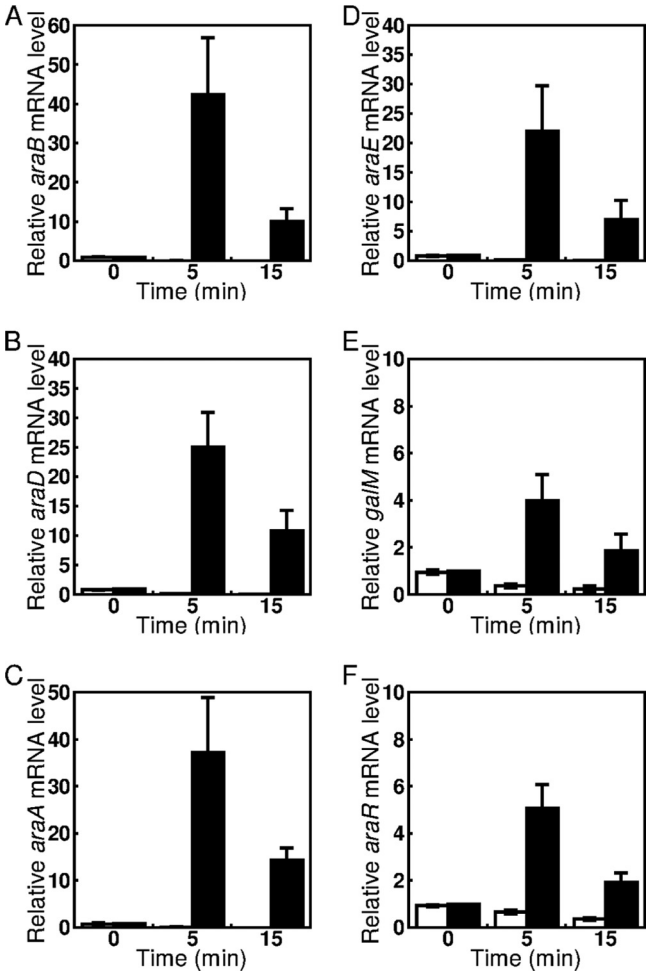

FIG 2 Changes in expression levels of *araB* (A), *araD* (B), *araA* (C), *araE* (D), *galM* (E), and *araR* (F) in response to L-arabinose. The *C. glutamicum* ATCC 31831 wild-type strain was grown in nutrient-rich A medium for 4 h, and exponentially growing cells were then supplemented with D-glucose (white bars) or L-arabinose (black bars) to a final concentration of 2% (wt/vol). The mRNA levels in cells incubated with sugars for 0, 5, and 15 min were determined by qRT-PCR and are presented relative to the value obtained for the cells before addition of L-arabinose. Mean values and standard deviations for at least three independent cultures are shown.

these genes within 15 min of addition of L-arabinose by using qRT-PCR. *C. glutamicum* ATCC 31831 wild-type cells were cultured in nutrient-rich A medium for 4 h, and exponentially growing cells were then supplemented with L-arabinose or D-glucose to a final concentration of 2% (wt/vol). The levels of *araB*, *araD*, and *araA* mRNAs increased >20-fold within 5 min and then decreased to some extent in the subsequent 10 min (Fig. 2A to C). The expression levels of these mRNAs after 15 min of incubation with L-arabinose were nearly 10-fold higher than their levels before L-arabinose addition. Thus, it is likely that *araB*, *araD*, and *araA* are transcribed as a tricistronic mRNA. A very similar expression pattern in response to L-arabinose was observed for *araE* (Fig. 2D). The expression levels of *galM* and *araR* mRNAs increased about 4-fold within 5 min and then decreased in the subsequent 10 min (Fig. 2E and F). The levels of these mRNAs 15 min after L-arabinose addition were 2-fold higher than their levels before the addition of L-arabinose. These results indicate that *galM* and *araR* are upregulated in response to L-arabinose, similar to the other genes in this cluster, but to a much smaller extent. Therefore, it is likely that *araE* is transcribed under the control of its own promoter, independent of the probable upstream *galM-araR* operon. Addition of D-glucose in place of L-arabinose resulted in a decrease in the expression levels of these five genes during 15 min of cultivation (Fig. 2A to F), and the effects of glucose on L-arabinose-inducible gene expression were examined further as described below.

Exponentially growing cells cultured in nutrient-rich A medium were supplemented with L-arabinose and/or D-glucose at 2% (wt/vol) (each) and then incubated for 60 min. Addition of D-glucose resulted in at least a 2-fold decrease in the levels of *araA* and *araE* mRNAs within 30 min, but after 60 min of incubation, these mRNAs reverted to their levels before the addition of D-glucose (Fig. 3). The prominent upregulation of *araA* and *araE* observed within 30 min of L-arabinose supplementation was strongly suppressed by supplementation of D-glucose simultaneously. However, the levels of these mRNAs were increased within 60 min of incubation in the presence of both L-arabinose and D-glucose, to the same levels as those in the presence of only L-arabinose.

Next, to confirm the involvement of AraR in L-arabinose-inducible gene expression, we compared the levels of *araB*, *araE*, and *galM* mRNAs in an *araR* deletion mutant strain (Δ*araR*) with those in the wild-type strain after 5 min of incubation in the presence or absence of L-arabinose. In the absence of L-arabinose, the expression level of *araB* in the Δ*araR* strain was 50-fold higher

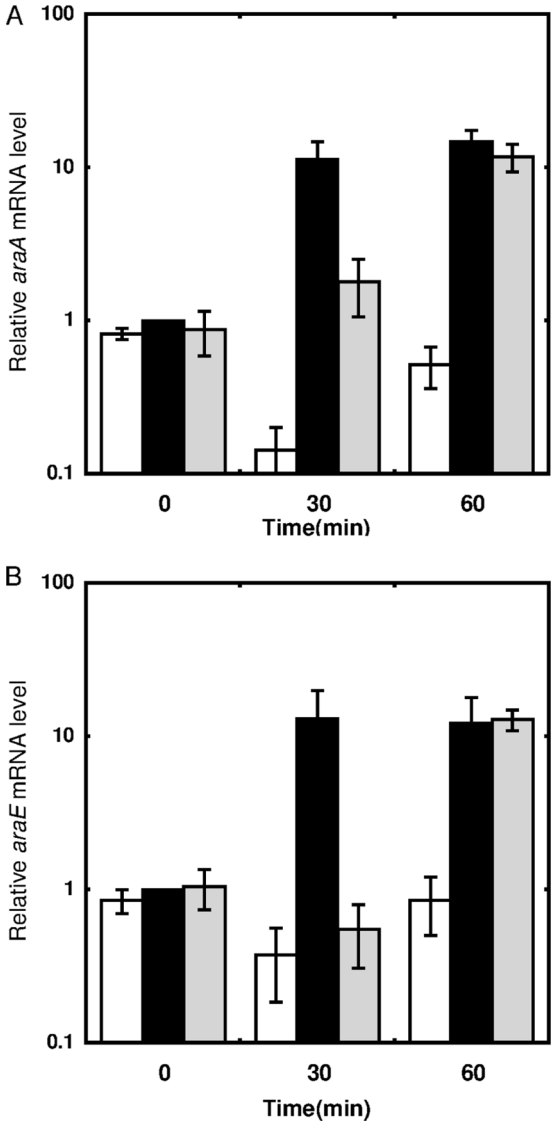

FIG 3 Effects of D-glucose on L-arabinose-dependent upregulation of *araA* (A) and *araE* (B). The *C. glutamicum* ATCC 31831 wild-type strain was grown in nutrient-rich A medium for 4 h, and exponentially growing cells were then supplemented with D-glucose (white bars), L-arabinose (black bars), or L-arabinose plus D-glucose (gray bars), to a final concentration of 2% (wt/vol) for each sugar. The mRNA levels in cells incubated with sugars for 0, 30, and 60 min were determined by qRT-PCR and are presented relative to the values obtained for the cells before addition of L-arabinose. Mean values and standard deviations for at least three independent cultures are shown.

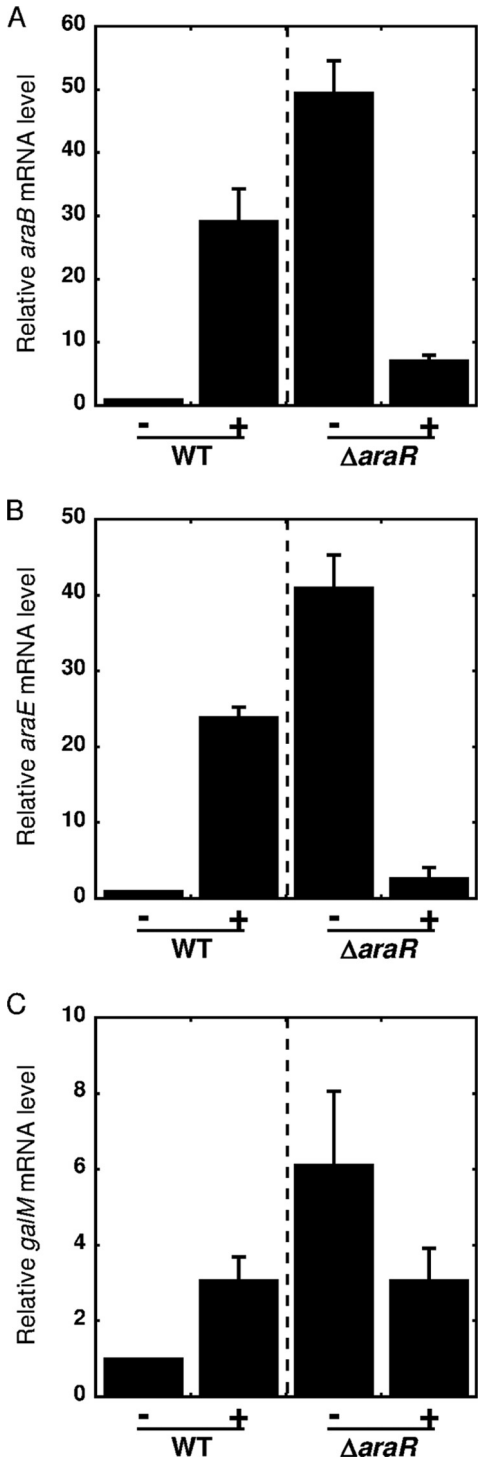

FIG 4 Effects of *araR* disruption on expression of *araB* (A), *araE* (B), and *galM* (C). The *C. glutamicum* ATCC 31831 wild-type strain (WT) and the *araR* deletion mutant strain (Δ*araR*) were grown in nutrient-rich A medium for 4 h, and exponentially growing cells were then supplemented with L-arabinose to a final concentration of 2% (wt/vol). The mRNA levels in cells incubated with L-arabinose for 0 min (−) or 5 min (+) were determined by qRT-PCR and are presented relative to the values obtained for the wild-type cells before addition of L-arabinose. Mean values and standard deviations for at least three independent cultures are shown.

than that in the wild-type strain (Fig. 4A). The level of *araB* mRNA in the Δ*araR* strain was even higher than the L-arabinose-induced level in the wild-type strain but decreased significantly in the presence of L-arabinose. The same results were observed for *araE* (Fig. 4B). Deletion of *araR* also resulted in an increase in the level of *galM* mRNA in the absence of L-arabinose, and the AraR-independent downregulation in response to L-arabinose was observed (Fig. 4C), but these changes in *galM* expression were much smaller than those in the case of *araB* and *araE*.

The AraR protein binds to the upstream regions of *araB*, *galM*, and *araE*. To confirm the ability of the AraR protein to bind to the upstream regions of the L-arabinose-inducible genes, EMSA

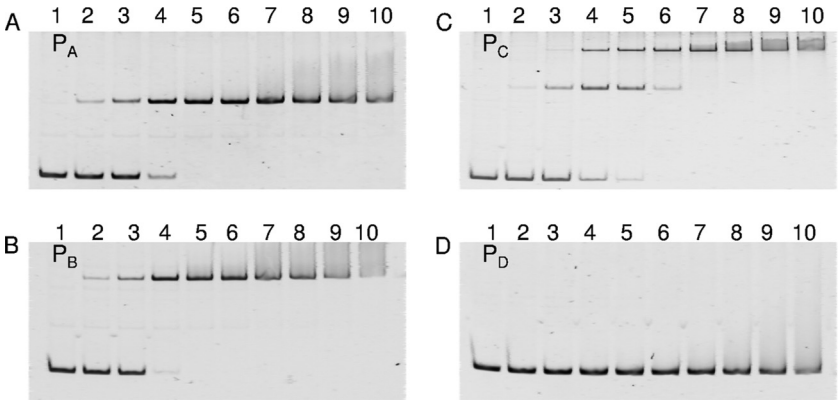

FIG 5 *In vitro* binding of AraR to the upstream regions of the L-arabinose utilization genes. EMSA was carried out with the AraR protein. The DNA probes used were the upstream regions of *araB* (P_A) (A), *galM* (P_B) (B), *araE* (P_C) (C), and *araR* (P_D) (D) shown in Fig. 1A. Each 20-μl binding reaction mixture, containing the DNA probe at 1 nM and the AraR protein at various concentrations (lanes 1, 0 nM; lanes 2, 10 nM; lanes 3, 25 nM; lanes 4, 50 nM; lanes 5, 75 nM; lanes 6, 100 nM; lane 7, 125 nM; lane 8, 150 nM; lane 9, 175 nM; and lane 10, 200 nM), was subjected to electrophoresis on a 6% polyacrylamide gel.

was performed using the *C. glutamicum* ATCC 31831 AraR protein, which was expressed in *E. coli* and purified. DNA fragments of 381 bp (P_A; between positions −353 and +28 with respect to the *araB* translation start site), 400 bp (P_B; between positions −380 and +20 with respect to the *galM* translation start site), 394 bp (P_C; between positions −364 and +30 with respect to the *araE* translation start site), and 399 bp (P_D; between positions −388 and +11 with respect to the *araR* translation start site) were used as DNA probes (Fig. 1A). The AraR protein reduced the electrophoretic mobility of the *araB* upstream DNA fragment; the amount of the AraR-DNA complex increased as the concentration of the AraR protein increased (Fig. 5A). The EMSAs also revealed that the AraR protein bound to the *galM* and *araE* upstream DNA fragments (Fig. 5B and C). In contrast, no complex of AraR with the *araR* upstream DNA fragment was detected (Fig. 5D). These results indicated that AraR binds to the upstream regions of *araB*, *galM*, and *araE* in a sequence-specific manner.

There was a 124-bp overlap between the upstream regions of *araB* and *galM* used for the DNA probes, each of which formed a complex with AraR as detected as a single shifted band (Fig. 5A and B). We performed EMSAs using a deletion series of the *galM* upstream region as DNA probes (Fig. 6A). The AraR protein was incubated with the respective DNA fragments and subjected to gel electrophoresis. The results of these EMSAs are shown in Fig. 6B;

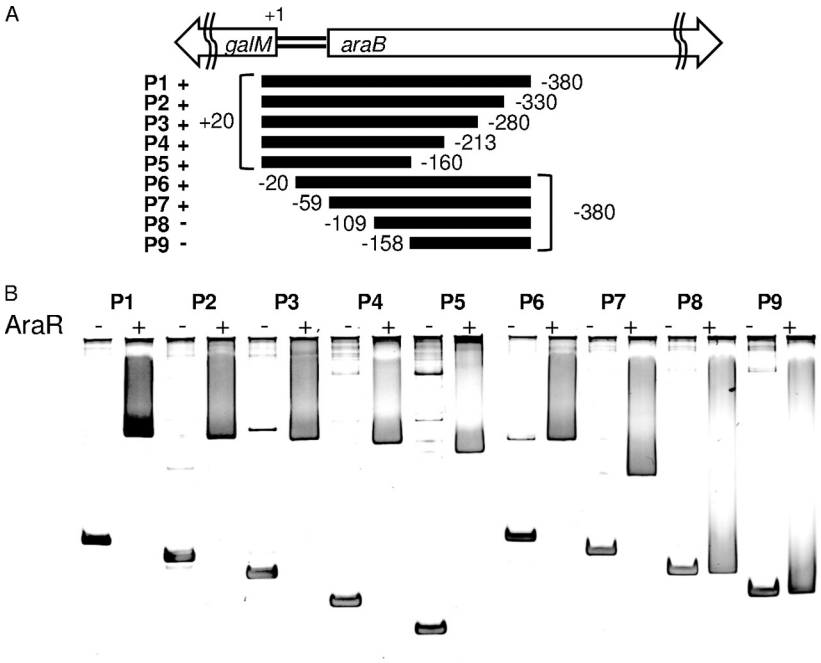

FIG 6 EMSA with the AraR protein and various 5′- and 3′-deletion fragments of the *galM* upstream region. (A) The DNA fragments (P1 to P9; P1 is the same as P_B in Fig. 1A and 5), with their EMSA results indicated (+, positive for binding of AraR; and −, no binding of AraR), corresponded to the regions located at the positions indicated with respect to the translation start site of *galM*. (B) Each 20-μl binding reaction mixture, containing 40 ng of the DNA probe with (+) or without (−) the AraR protein at 100 nM, was subjected to electrophoresis on a 6% polyacrylamide gel.

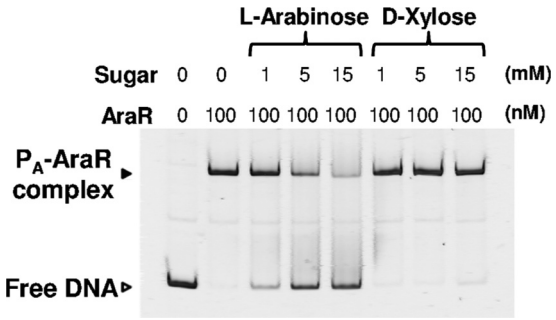

FIG 7 Effects of L-arabinose and D-xylose on the DNA-binding activity of AraR. EMSA was carried out with the AraR protein and the *araB* upstream region. Each 20-μl binding reaction mixture, containing the DNA probe P_A (Fig. 5) at 1 nM with or without the AraR protein at 100 nM, was subjected to electrophoresis on a 6% polyacrylamide gel. The reaction mixture was supplemented with L-arabinose or D-xylose at various concentrations (1, 5, and 15 mM). The free DNA and the DNA-protein complex are indicated by white and black arrowheads, respectively.

positive or no binding of AraR is indicated by + or −, respectively, in Fig. 6A. The EMSAs revealed that the AraR protein bound to all these 5′-deletion fragments (P2, P3, P4, and P5 in Fig. 6). A shifted band corresponding to a complex with AraR was observed for two of the 3′-deletion fragments (P6 and P7 in Fig. 6), but not for the other two (P8 and P9 in Fig. 6). These results indicate that the AraR-binding site upstream of *galM* is located within the 50-bp region between positions −59 and −109 with respect to the translation start site of *galM*. This AraR-binding site is also included in the *araB* upstream DNA fragment (P_A) used for the EMSAs described above.

AraR-binding activity is reduced by L-arabinose. The effects of L-arabinose on the DNA-binding activity of the AraR protein were examined by EMSA (Fig. 7). At a concentration of 5 mM, L-arabinose clearly reduced the formation of the complex of AraR with the *araB* upstream DNA fragment. In contrast, D-xylose did not affect the DNA-binding activity of AraR even at 15 mM. The binding of AraR to the *araB* upstream DNA fragment was inhibited as a function of the concentration of L-arabinose, from 1 mM to 15 mM. These results indicate that L-arabinose acts as a negative effector of the binding activity of AraR.

Identification of AraR-binding sites in the region upstream of *araB* and *araE*. The transcription start sites of the *araB*, *araE*, and *galM* genes determined by 5′-RACE were located 7, 104, and 69 bp upstream of the respective translation start sites (Fig. 1B and C). The consensus sequence of the −10 region of *C. glutamicum* SigA-dependent promoters (33) is found in their 5′-upstream regions. The consensus sequence of the −35 region is found in the *araB* and *araE* promoters but not in the *galM* promoter.

Next, we performed DNase I footprinting analysis to determine the AraR-binding site within the intergenic region between *araB* and *galM* (Fig. 8A). The DNA region protected against DNase I digestion by the binding of AraR was identified between positions +4 and +28 with respect to the transcription start site of *araB*. This protected region also corresponds to the region between positions −5 and −29 with respect to the transcription start site of *galM*, which is located divergently from that of *araB* (Fig. 1B and 8A). Therefore, the single AraR-binding site overlaps not only the translation start site of *araB* but also the −10 region of the divergent *galM* promoter. This AraR-binding site is included in the overlap between the *araB* and *galM* upstream DNA fragments used in the EMSAs described earlier (Fig. 5A and B).

DNase I footprinting analysis using the *araE* promoter region revealed that the AraR protein protects two regions: one between positions +5 and +28 and one between positions −28 and −46 with respect to the transcription start site of *araE* (Fig. 1C and 8B). This is consistent with the results of EMSA using the *araE* promoter region, which showed two shifted bands corresponding to complexes with AraR (Fig. 5C). These results indicate that one of the two AraR-binding sites corresponds to the 5′-untranslated region and the other overlaps the −35 region of the *araE* promoter (Fig. 1C).

We found the consensus sequence ATGTtAGCGnTaAcat based on alignment of the three AraR-binding sites: one in the *araB* upstream region and two in the *araE* upstream region (Fig. 9A). This 16-bp consensus sequence has an imperfect inverted repeat of the 8-bp element. The palindromic sequence has some similarity to the operator sequences of the LacI/GalR family transcriptional regulators (34, 35). Effects of mutations in the putative recognition sequence in the *araB* upstream region on the binding of AraR were examined by EMSAs (Fig. 9B to H). Three consecutive base pairs at various positions within the 16-bp consensus sequence were mutated, and the resulting *araB* upstream fragments carrying the mutations (mut1 to mut6 in Fig. 9B) were used as DNA probes in EMSAs. The EMSAs revealed that the AraR protein bound to the mut1 and mut6 fragments (Fig. 9C and H) with the same affinity as that for the wild-type *araB* upstream fragment, as shown earlier (Fig. 5A). In contrast, lower binding affinities were observed for the mut2, mut3, and mut5 fragments (Fig. 9D, E, and G), and no binding of AraR to the mut4 fragment was detected at all (Fig. 9F). These results indicate that the CG nucleotides located in the center of the palindrome are essential for the binding of AraR and that the nucleotides located in the 5′ half of the palindrome are more important for AraR binding than those of the 3′ half, consistent with the conservation between the target sequences identified here.

L-Arabinose-dependent induction of AraR-mediated gene expression is independent of catabolism of L-arabinose. The role of L-arabinose catabolism in the L-arabinose-dependent induction of gene expression was examined by qRT-PCR, using the *araA*, *araB*, and *araD* deletion mutants (Δ*araA*, Δ*araB*, and Δ*araD* strains, respectively). Previously, we reported that these deletion mutants cannot grow on L-arabinose as the sole carbon source (26). For qRT-PCR analysis, these strains were grown in nutrient-rich A medium, and then exponentially growing cells were supplemented with 2% (wt/vol) L-arabinose, as described earlier. In the absence of L-arabinose, the level of *araB* mRNA in the Δ*araA* and Δ*araD* strains was the same as that in the wild-type strain. Within 5 min of L-arabinose supplementation, *araB* mRNA was markedly upregulated in the Δ*araA* and Δ*araD* strains, to an extent comparable to that in the case of the wild-type strain (Fig. 10A). However, in the subsequent 10 min, the expression level of *araB* remained high in both of the deletion mutants, in contrast to the decreased level in the wild-type strain described earlier. Similar results were observed for *araA* expression in either the Δ*araB* (Fig. 10A) or Δ*araD* (data not shown) strain, and the same was observed for *araD* expression in either the Δ*araA* or Δ*araB* strain (data not shown). Furthermore, the expression of *araE* and *araR* was also upregulated within 5 min of L-arabinose supplementation in each of the Δ*araA*, Δ*araB*, and Δ*araD* strains, as in the

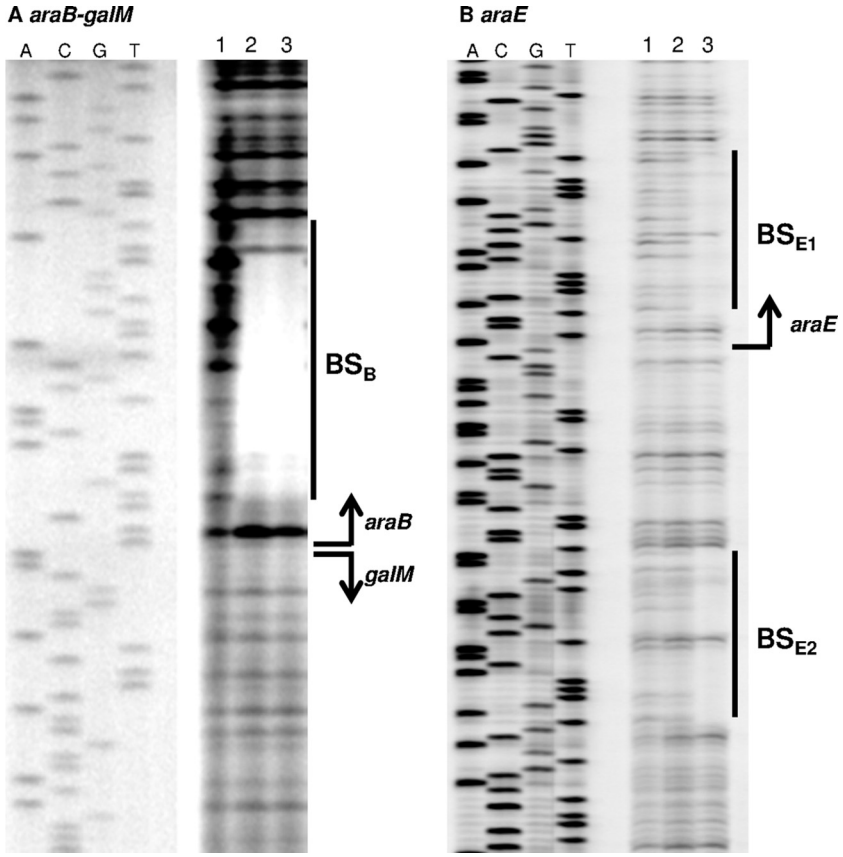

FIG 8 DNase I footprinting analysis with the AraR protein and the *araB-galM* intergenic region (A) or the *araE* upstream region (B). Each 20-μl binding reaction mixture, containing 80 ng (A) or 120 ng (B) of the DNA probe and the AraR protein at various concentrations (in panel A, lane 1, no protein; lane 2, 500 nM; and lane 3, 1,000 nM; and in panel B, lane 1, no protein; lane 2, 600 nM; and lane 3, 1,300 nM), was subjected to DNase I treatment followed by electrophoresis on a 5.5% sequencing gel. The sequence of each of these DNA regions was determined with the same labeled primer and plasmid as those for the footprinting probe and is shown to the left. The protected region and the transcription start site are indicated by a solid line and a bent arrow, respectively.

wild-type strain (Fig. 10B and C). After induction, the expression levels of these genes in the mutant strains hardly decreased in the subsequent 10 min, in contrast to the case in the wild-type strain. These results indicate that catabolism of L-arabinose taken up by cells is not required for the L-arabinose-dependent induction of these genes under the control of AraR but is required for the subsequent downregulation observed for the wild-type strain.

Upregulation of L-arabinose utilization genes is dependent on uptake of L-arabinose. Previously, we reported that deletion of *araE*, encoding an L-arabinose transporter, causes a low growth rate and reduces the final cell density in the presence of a low concentration of L-arabinose (26). Effects of different concentrations of L-arabinose on *araB*, *araE*, and *araR* expression were examined using the *araE* deletion mutant strain (Δ*araE*) and the wild-type strain. Exponentially growing cells cultured in nutrient-rich A medium were supplemented with L-arabinose to final concentrations of 0.5 mM, 5 mM, 50 mM, and 200 mM and subsequently incubated for 5 min (Fig. 11). qRT-PCR analysis revealed that before the supplementation of L-arabinose, there was no apparent difference in the expression levels of each of the *araB*, *araE*, and *araR* genes between the Δ*araE* strain and the wild-type strain. The expression of *araE* was detected using a pair of qRT-PCR primers corresponding to a region upstream of the deleted region in the Δ*araE* strain. With the wild-type strain, lower concentrations of L-arabinose tended to be more effective for induction of the *araB* and *araE* genes. The expression of these genes was also markedly induced by L-arabinose at concentrations of 5 mM, 50 mM, and 200 mM in the Δ*araE* strain, to an extent comparable to that in the case of the wild-type strain. However, addition of 0.5 mM L-arabinose was much less effective for induction of the expression of *araB* and *araE* in the Δ*araE* strain than in the wild-type strain. Similar effects of *araE* inactivation on L-arabinose-dependent induction were observed for *araR*, although its dose response to L-arabinose in the wild-type strain was minimal, in contrast to the case of *araB* and *araE* as described above (Fig. 11). These results indicate that the uptake of L-arabinose is required for the induction of these genes under the control of AraR.

DISCUSSION

In this study, we showed that the *C. glutamicum* ATCC 31831 AraR protein, belonging to the LacI family of transcriptional regulators, binds to the *araB* and *araE* upstream regions and that the *in vitro* DNA-binding activity of AraR is reduced by L-arabinose. We previously reported that the expression of *araBDA* and *araE* is upregulated in the presence of L-arabinose compared to that in the presence of D-glucose and by inactivation of *araR* in the absence of L-arabinose (26). These results indicate that AraR acts as a transcriptional repressor of the L-arabinose catabolic and uptake genes

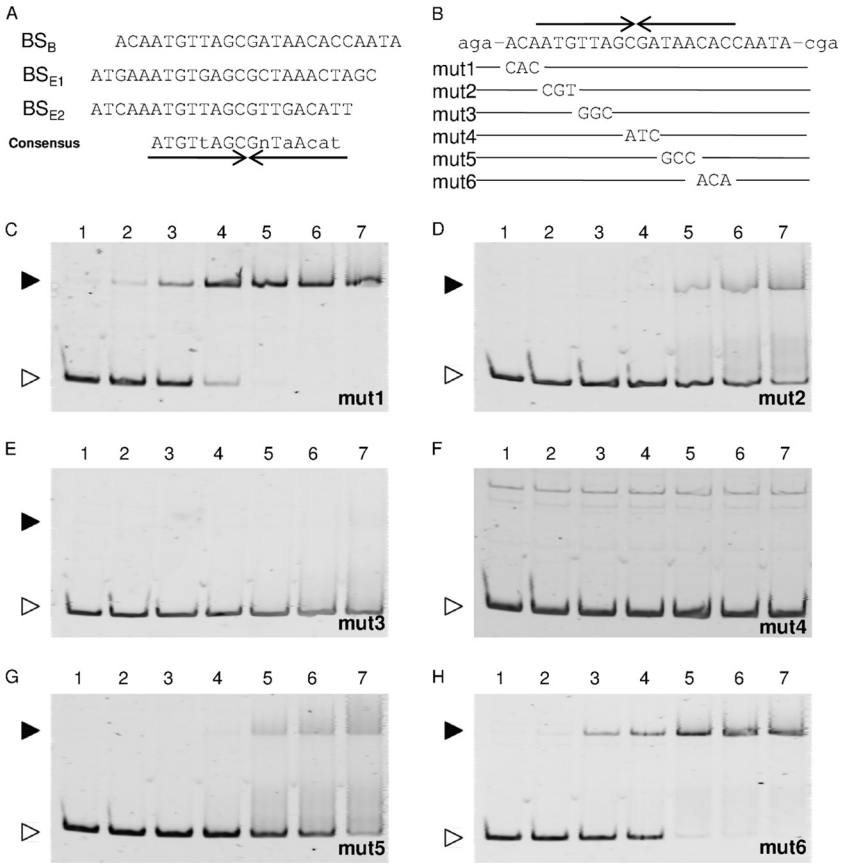

FIG 9 The AraR-binding motif. (A) The three AraR-binding sites determined by DNase I footprinting analyses, located in the *araB* (BS_B) and *araE* (BS_{E1} and BS_{E2}) upstream regions, are aligned with the consensus sequence, in which the nucleotides in capital and lowercase letters are identical in all and two of the three sites, respectively, and "n" stands for any nucleotide. Various mutations (mut1 to mut6) were introduced into the AraR-binding site within the *araB* upstream region (B) and used for EMSAs (C to H). An inverted repeat sequence is indicated by arrows under (A) or above (B) the sequence. Each 20-μl binding reaction mixture, containing the DNA probe at 1 nM and the AraR protein at various concentrations (lanes 1, no protein; lanes 2, 10 nM; lanes 3, 25 nM; lanes 4, 50 nM; lanes 5, 75 nM; lanes 6, 100 nM; and lanes 7, 125 nM), was subjected to electrophoresis on a 6% polyacrylamide gel. The free DNA and the DNA-protein complex are indicated by white and black arrowheads, respectively.

(*araBDA* and *araE*) and that AraR-mediated expression is derepressed in the presence of L-arabinose. This is consistent with the rapid induction of these genes after supplementation with L-arabinose, as shown in this study. It should be noted that L-arabinose induced these genes under the control of AraR in mutant strains deficient in L-arabinose catabolism, as well as in the wild-type strain (Fig. 10). Furthermore, in the strain deficient in *araE*, encoding an L-arabinose transporter, the induction of these genes by a low concentration of L-arabinose was markedly suppressed (Fig. 11), suggesting that the uptake of L-arabinose is required for the derepression of AraR-mediated gene expression. It is noted that the inactivation of *araE* results in slow growth with a low concentration of L-arabinose as the sole carbon source (26). However, this mutant strain grows as well as the wild-type strain in the presence of a high concentration of L-arabinose (26), which is consistent with the high induction levels of the L-arabinose utilization genes shown in this study. Taken together, our data establish that L-arabinose acts as an efficient inducer of AraR-mediated gene expression in *C. glutamicum* ATCC 31831 *in vivo*.

As in *C. glutamicum* ATCC 31831, L-arabinose plays a key role in the regulation of L-arabinose utilization genes as a primary inducer in *E. coli* and *B. subtilis* (36–39). However, their L-arabinose-responsive transcriptional regulators belong to distinct families. The AraC protein in *E. coli* functions as an activator in the presence of L-arabinose but as a repressor in the absence of L-arabinose (13, 14, 16). In *B. subtilis*, the oligomerization and effector-binding domains, located in the C terminus of the L-arabinose-responsive transcriptional regulator AraR, have sequence similarity to the LacI/GalR family proteins, but the DNA-binding domain, located in the N terminus, has sequence similarity to members of the GntR family (21, 40). As shown in this study, the LacI-type transcriptional regulator AraR in *C. glutamicum* ATCC 31831 recognizes a 16-bp palindromic sequence which has common features of the recognition sequences described so far for the other members of this family (35). It has been reported that the LacI/GalR family members coordinate available nutrients with expression of catabolic genes, but some regulate processes as diverse as nucleotide biosynthesis and toxin expression (41–43). As established for this family of transcriptional regulators, it is likely that binding of L-arabinose to the C-terminal domain in the *C. glutamicum* ATCC 31831 AraR protein results in a conformational change in the N-terminal DNA-binding domain. Thereby, the DNA-binding activity is inhibited in the presence of L-arabinose. This is the first report of the L-arabinose-dependent changes in

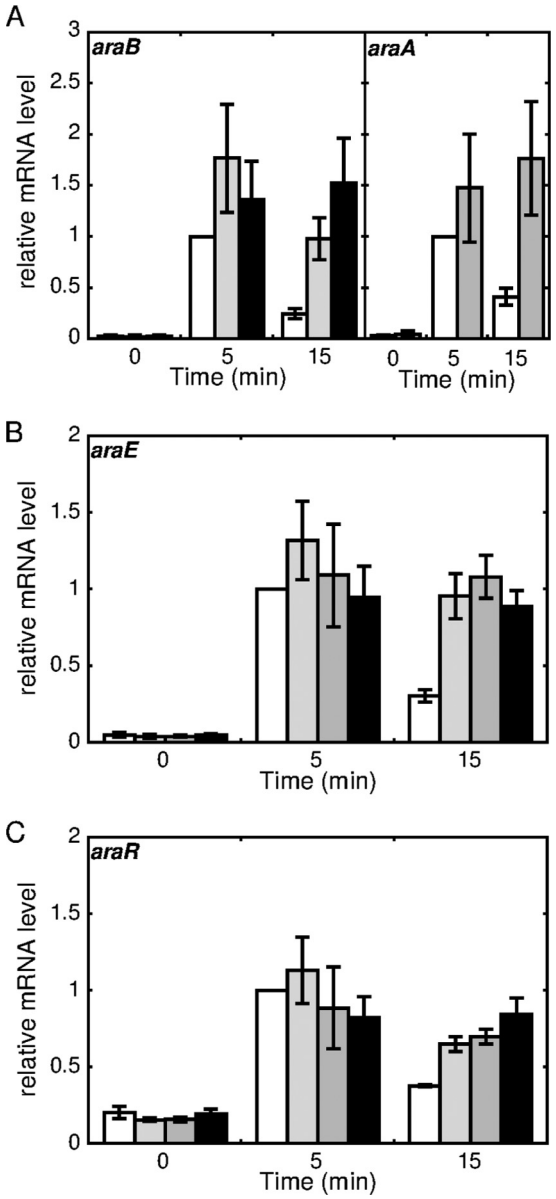

FIG 10 Effects of disruption of L-arabinose catabolic genes on L-arabinose-inducible gene expression. The *C. glutamicum* ATCC 31831 wild-type (white bars), *araA* (gray bars), *araB* (dark gray bars), and *araD* (black bars) deletion mutant strains were grown in nutrient-rich A medium for 4 h, and exponentially growing cells were then supplemented with L-arabinose at a final concentration of 2% (wt/vol). The levels of *araB* and *araA* (A), *araE* (B), and *araR* (C) in cells incubated with L-arabinose for 0, 5, and 15 min were determined by qRT-PCR. The mRNA levels are presented relative to the values obtained for wild-type cells incubated with L-arabinose for 5 min. Mean values and standard deviations for at least three independent cultures are shown.

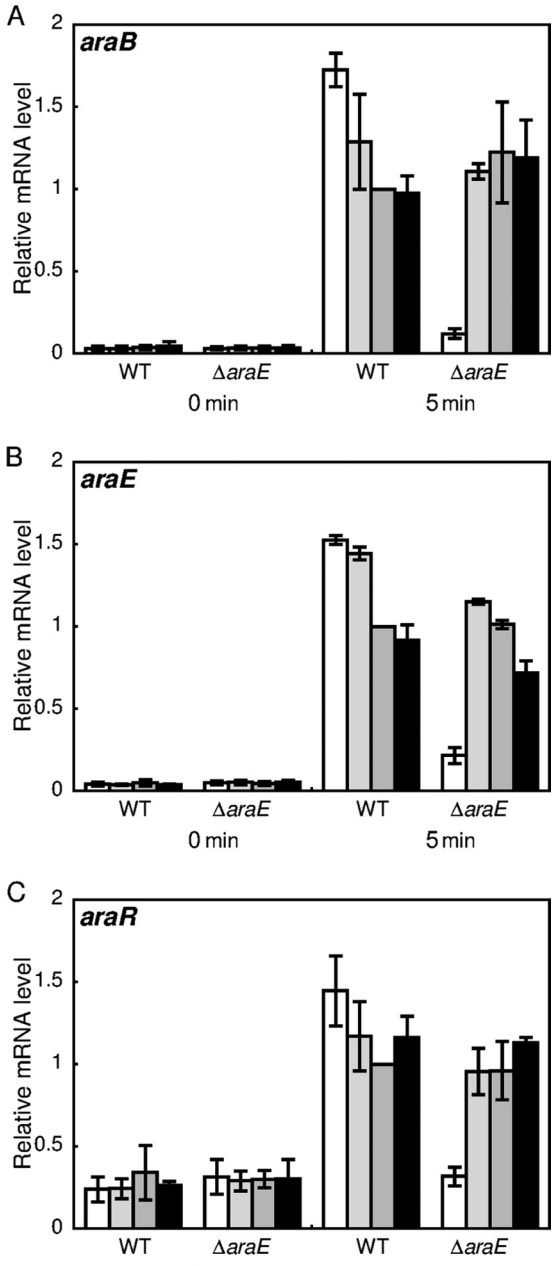

FIG 11 Effects of *araE* disruption on L-arabinose-dependent upregulation of *araB* (A), *araE* (B), and *araR* (C). The *C. glutamicum* ATCC 31831 wild-type (WT) and *araE* deletion mutant (Δ*araE*) strains were grown in nutrient-rich A medium for 4 h, and exponentially growing cells were then supplemented with L-arabinose to final concentrations of 0.5 mM (white bars), 5 mM (light gray bars), 50 mM (dark gray bars), and 200 mM (black bars). The mRNA levels in the cells incubated with L-arabinose for 0 and 5 min were determined by qRT-PCR and are presented relative to the values obtained for wild-type cells incubated with 50 mM L-arabinose. Mean values and standard deviations for at least three independent cultures are shown.

DNA-binding activity of the LacI/GalR family regulators. The gene cluster associated with L-arabinose utilization has never been found in other strains of *C. glutamicum* so far. However, *Mycobacterium smegmatis* and *Streptomyces venezuelae* have homologs of the L-arabinose catabolic genes of *C. glutamicum* ATCC 31831, and LacI-type transcriptional regulators encoded near these genes on their chromosomes have 44% and 41% amino acid sequence identity, respectively, to *C. glutamicum* ATCC 31831 AraR. In *M. smegmatis*, which is phylogenetically related to *C. glutamicum*, the L-arabinose catabolic genes are upregulated by L-arabinose (44, 45), but the involvement of the AraR homolog in this regulation remains to be investigated. Many other strains of actinobacteria identified to date lack the L-arabinose catabolic genes, but the

AraR-dependent regulatory system of L-arabinose utilization reported here might be conserved among other strains that have yet to be unidentified.

On the chromosome of *C. glutamicum* ATCC 31831, the *araR* gene is located immediately downstream of *galM*, encoding a putative aldose 1-epimerase. We showed here that *araR* and *galM* are similarly upregulated by L-arabinose. These results suggest that *araR* is cotranscribed with *galM* under the control of the L-arabinose-inducible *galM* promoter. Since the AraR-binding site is located within the intergenic region between *araB* and *galM* (Fig. 1), AraR may regulate the expression of the divergently transcribed *araBDA* and *galM-araR* genes through the same mechanism. However, L-arabinose supplementation and/or inactivation of *araR* showed much smaller effects on the expression of *galM* and *araR* than the effects on the expression of *araBDA* (Fig. 4). The AraR-binding site in the *galM* promoter overlaps its −10 region (Fig. 1) but is located downstream of the transcription start site of *araB*. The difference in the location of the AraR-binding site may explain the different degree of transcriptional repression. Furthermore, our previous study showed that the expression of *araR*, but not that of *galM*, is upregulated in cells grown in minimal medium with L-arabinose as the sole carbon source compared to that in cells grown on D-glucose (26). Thus, it is possible that the *araR* gene is under the control of its own promoter, although no binding of AraR to the *araR* upstream region was observed (Fig. 5). Further studies are needed to elucidate the role of AraR in *galM* promoter activity. The other regulatory mechanism independent of AraR should also be considered.

We found the 16-bp consensus palindromic sequence ATGTtAGCGnTaAcat based on the alignment of the three binding sites of *C. glutamicum* ATCC 31831 AraR identified here. This motif is present between positions +8 and +23 bp with respect to the *araB* transcription start site and in two positions, one between positions +10 and +25 bp and the other between positions −29 and −44 bp, with respect to the *araE* transcription start site (Fig. 1). Tremendous efforts have been devoted to characterizing the LacI/GalR transcriptional regulators so far, and the results indicate that a few bases in the center of the palindromic structure are strictly conserved among the members of this family (34, 35). However, the peripheral nucleotides in the palindrome are less conserved, which allows these regulators to specifically recognize their own target sequences. The consensus sequence of the multiple *C. glutamicum* ATCC 31831 AraR-binding sites has CG in the center of the palindrome, which is conserved among the LacI/GalR family members. As expected, the introduction of mutations in the center of the palindromic sequence resulted in no formation of a complex with AraR (Fig. 9F). The AraR-binding site is located downstream of the transcription start sites of *araB* and *araE*, suggesting that binding of AraR prevents the elongation of transcription by RNA polymerase. It is noted that a loop structure formed by the cooperative binding of a transcriptional repressor to separate sites in its target promoter is one of the most efficient mechanisms to repress transcription, as described for the LacI family (e.g., LacI and GalR in *E. coli*) (46, 47). This was also reported for the L-arabinose utilization genes under the control of different types of L-arabinose-responsive transcriptional regulators, i.e., AraC in *E. coli* (12) and AraR in *B. subtilis* (19). It is interesting that the second distal binding site of *C. glutamicum* ATCC 31831 AraR overlaps the −35 region of the L-arabinose transporter gene *araE* promoter, with a 37-bp space from its proximal binding site (Fig. 1C). This distal site may act cooperatively with the proximal one, although binding of AraR to the former site may prevent RNA polymerase from the interaction with the promoter, irrespective of the latter site. In this context, it should be noted that no similar distal AraR-binding site was found in the upstream region of the L-arabinose catabolic genes *araBDA*.

We found that in the *araR* deletion mutant strain, the expression levels of *araB* and *araE* markedly decreased within 5 min of the addition of L-arabinose, although in the absence of L-arabinose these mRNAs in this mutant were significantly elevated compared with those in the wild-type strain (Fig. 4). This AraR-independent regulatory mechanism may be involved in the decline after the rapid induction of the *araBDA* operon and *araE* within 15 min of the addition of L-arabinose to the wild-type cells (Fig. 2), although fluctuation of the intracellular level of L-arabinose may also affect the AraR-dependent expression of these genes in the beginning of L-arabinose catabolism. In *E. coli*, the induction of the L-arabinose uptake and catabolic genes was followed by repression during 1 h of incubation with L-arabinose (16). It has been suggested that the cAMP-dependent transcriptional regulator CRP is responsible for the L-arabinose-dependent repression. The expression of the L-arabinose-inducible genes in *E. coli* is severely inhibited in the presence of D-glucose, through a carbon catabolite repression mechanism mediated by CRP (48–50). We found that the L-arabinose catabolic and uptake genes in *C. glutamicum* ATCC 31831 were transiently downregulated upon the addition of D-glucose (Fig. 3). Moreover, the addition of L-arabinose plus D-glucose resulted in a delay of the upregulation of L-arabinose-inducible gene expression (Fig. 3). However, the steady-state expression level upregulated in the presence of both L-arabinose and D-glucose was comparable to that in the presence of only L-arabinose. This is consistent with our previous study showing the simultaneous utilization of L-arabinose and D-glucose, at the same rates (26). It should be noted that the global carbon catabolite repression system, mediated by CRP and CcpA in *E. coli* and *B. subtilis*, respectively, has never been found in *C. glutamicum*. Moreover, *C. glutamicum* has the ability to consume various carbon sources simultaneously (51–54). At present, a search for another transcriptional regulator involved in the probable carbon source-responsive multilayered control of the L-arabinose utilization genes is under way in our laboratory. An understanding of this regulatory system of L-arabinose utilization genes in *C. glutamicum* ATCC 31831 should provide critical insights into the unique control of genes involved in sugar metabolism in this industrially useful microorganism and a basis for the development of efficient bioprocesses.

ACKNOWLEDGMENTS

We thank Crispinus A. Omumasaba (RITE) for critically reading the manuscript.

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

REFERENCES

1. **Yukawa H, Inui M, Vertès AA.** 2007. Genomes and genome-level engineering of amino acid-producing bacteria, p 349–401. *In* Wendisch VF (ed), Amino acid biosynthesis—pathways, regulation and metabolic engineering, vol 5. Springer, Berlin, Germany.
2. **Sahm H, Eggeling L, Eikmanns B, Krämer R.** 1995. Metabolic design in amino acid producing bacterium *Corynebacterium glutamicum*. FEMS Microbiol. Rev. **16:**243–252. http://dx.doi.org/10.1111/j.1574-6976.1995.tb00171.x.

3. **Inui M, Kawaguchi H, Murakami S, Vertès AA, Yukawa H.** 2004. Metabolic engineering of *Corynebacterium glutamicum* for fuel ethanol production under oxygen-deprivation conditions. J. Mol. Microbiol. Biotechnol. **8:**243–254. http://dx.doi.org/10.1159/000086705.
4. **Jojima T, Inui M, Yukawa H.** 2013. Biorefinery applications of *Corynebacterium glutamicum*, p 149–172. *In* Yukawa H, Inui M (ed), *Corynebacterium glutamicum*, vol 23. Springer, Berlin, Germany.
5. **Okino S, Inui M, Yukawa H.** 2005. Production of organic acids by Corynebacterium glutamicum under oxygen deprivation. Appl. Microbiol. Biotechnol. **68:**475–480. http://dx.doi.org/10.1007/s00253-005-1900-y.
6. **Aristidou A, Penttila M.** 2000. Metabolic engineering applications to renewable resource utilization. Curr. Opin. Biotechnol. **11:**187–198. http://dx.doi.org/10.1016/S0958-1669(00)00085-9.
7. **Elander RT, Dale BE, Holtzapple M, Ladisch MR, Lee YY, Mitchinson C, Saddler JN, Wyman CE.** 2009. Summary of findings from the Biomass Refining Consortium for Applied Fundamentals and Innovation (CAFI): corn stover pretreatment. Cellulose **16:**649–659. http://dx.doi.org/10.1007/s10570-009-9308-y.
8. **Sasaki M, Jojima T, Kawaguchi H, Inui M, Yukawa H.** 2009. Engineering of pentose transport in *Corynebacterium glutamicum* to improve simultaneous utilization of mixed sugars. Appl. Microbiol. Biotechnol. **85:**105–115. http://dx.doi.org/10.1007/s00253-009-2065-x.
9. **Kawaguchi H, Vertès AA, Okino S, Inui M, Yukawa H.** 2006. Engineering of a xylose metabolic pathway in *Corynebacterium glutamicum*. Appl. Environ. Microbiol. **72:**3418–3428. http://dx.doi.org/10.1128/AEM.72.5.3418-3428.2006.
10. **Sasaki M, Jojima T, Inui M, Yukawa H.** 2008. Simultaneous utilization of D-cellobiose, D-glucose, and D-xylose by recombinant *Corynebacterium glutamicum* under oxygen-deprived conditions. Appl. Microbiol. Biotechnol. **81:**691–699. http://dx.doi.org/10.1007/s00253-008-1703-z.
11. **Kawaguchi H, Sasaki M, Vertès AA, Inui M, Yukawa H.** 2008. Engineering of an L-arabinose metabolic pathway in *Corynebacterium glutamicum*. Appl. Microbiol. Biotechnol. **77:**1053–1062. http://dx.doi.org/10.1007/s00253-007-1244-x.
12. **Schleif R.** 2010. AraC protein, regulation of the L-arabinose operon in *Escherichia coli*, and the light switch mechanism of AraC action. FEMS Microbiol. Rev. **34:**779–796. http://dx.doi.org/10.1111/j.1574-6976.2010.00226.x.
13. **Lee NL, Gielow WO, Wallace RG.** 1981. Mechanism of *araC* autoregulation and the domains of two overlapping promoters, P_C and P_{BAD}, in the L-arabinose regulatory region of *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. A. **78:**752–756. http://dx.doi.org/10.1073/pnas.78.2.752.
14. **Hahn S, Schleif R.** 1983. *In vivo* regulation of the *Escherichia coli araC* promoter. J. Bacteriol. **155:**593–600.
15. **Seedorff J, Schleif R.** 2011. Active role of the interdomain linker of AraC. J. Bacteriol. **193:**5737–5746. http://dx.doi.org/10.1128/JB.05339-11.
16. **Johnson CM, Schleif RF.** 1995. *In vivo* induction kinetics of the arabinose promoters in *Escherichia coli*. J. Bacteriol. **177:**3438–3442.
17. **Inácio JM, Sá-Nogueira I.** 2008. Characterization of *abn2* (*yxiA*), encoding a *Bacillus subtilis* GH43 arabinanase, Abn2, and its role in arabino-polysaccharide degradation. J. Bacteriol. **190:**4272–4280. http://dx.doi.org/10.1128/JB.00162-08.
18. **Raposo MP, Inácio JM, Mota LJ, Sá-Nogueira I.** 2004. Transcriptional regulation of genes encoding arabinan-degrading enzymes in *Bacillus subtilis*. J. Bacteriol. **186:**1287–1296. http://dx.doi.org/10.1128/JB.186.5.1287-1296.2004.
19. **Mota LJ, Tavares P, Sá-Nogueira I.** 1999. Mode of action of AraR, the key regulator of L-arabinose metabolism in *Bacillus subtilis*. Mol. Microbiol. **33:**476–489. http://dx.doi.org/10.1046/j.1365-2958.1999.01484.x.
20. **Franco IS, Mota LJ, Soares CM, Sá-Nogueira I.** 2006. Functional domains of the *Bacillus subtilis* transcription factor AraR and identification of amino acids important for nucleoprotein complex assembly and effector binding. J. Bacteriol. **188:**3024–3036. http://dx.doi.org/10.1128/JB.188.8.3024-3036.2006.
21. **Franco IS, Mota LJ, Soares CM, Sá-Nogueira I.** 2007. Probing key DNA contacts in AraR-mediated transcriptional repression of the *Bacillus subtilis* arabinose regulon. Nucleic Acids Res. **35:**4755–4766. http://dx.doi.org/10.1093/nar/gkm509.
22. **Mota LJ, Sarmento LM, Sá-Nogueira I.** 2001. Control of the arabinose regulon in *Bacillus subtilis* by AraR in vivo: crucial roles of operators, cooperativity, and DNA looping. J. Bacteriol. **183:**4190–4201. http://dx.doi.org/10.1128/JB.183.14.4190-4201.2001.
23. **Krispin O, Allmansberger R.** 1998. The *Bacillus subtilis araE* protein displays a broad substrate specificity for several different sugars. J. Bacteriol. **180:**3250–3252.
24. **Hahn S, Dunn T, Schleif R.** 1984. Upstream repression and CRP stimulation of the *Escherichia coli* L-arabinose operon. J. Mol. Biol. **180:**61–72. http://dx.doi.org/10.1016/0022-2836(84)90430-3.
25. **Inácio JM, Costa C, Sá-Nogueira I.** 2003. Distinct molecular mechanisms involved in carbon catabolite repression of the arabinose regulon in *Bacillus subtilis*. Microbiology **149:**2345–2355. http://dx.doi.org/10.1099/mic.0.26326-0.
26. **Kawaguchi H, Sasaki M, Vertès AA, Inui M, Yukawa H.** 2009. Identification and functional analysis of the gene cluster for L-arabinose utilization in *Corynebacterium glutamicum*. Appl. Environ. Microbiol. **75:**3419–3429. http://dx.doi.org/10.1128/AEM.02912-08.
27. **Sambrook J, Russell DW.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
28. **Studier FW, Moffatt BA.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189:**113. http://dx.doi.org/10.1016/0022-2836(86)90385-2.
29. **Inui M, Murakami S, Okino S, Kawaguchi H, Vertès AA, Yukawa H.** 2004. Metabolic analysis of *Corynebacterium glutamicum* during lactate and succinate productions under oxygen deprivation conditions. J. Mol. Microbiol. Biotechnol. **7:**182–196. http://dx.doi.org/10.1159/000079827.
30. **Vertès AA, Inui M, Kobayashi M, Kurusu Y, Yukawa H.** 1993. Presence of *mrr*- and *mcr*-like restriction systems in coryneform bacteria. Res. Microbiol. **144:**181–185. http://dx.doi.org/10.1016/0923-2508(93)90043-2.
31. **Sanger F, Nicklen S, Coulson AR.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U. S. A. **74:**5463–5467. http://dx.doi.org/10.1073/pnas.74.12.5463.
32. **Okibe N, Suzuki N, Inui M, Yukawa H.** 2011. Efficient markerless gene replacement in *Corynebacterium glutamicum* using a new temperature-sensitive plasmid. J. Microbiol. Methods **85:**155–163. http://dx.doi.org/10.1016/j.mimet.2011.02.012.
33. **Pátek M, Nešvera J.** 2011. Sigma factors and promoters in *Corynebacterium glutamicum*. J. Biotechnol. **154:**101–113. http://dx.doi.org/10.1016/j.jbiotec.2011.01.017.
34. **Camas FM, Alm EJ, Poyatos JF.** 2010. Local gene regulation details a recognition code within the LacI transcriptional factor family. PLoS Comput. Biol. **6:**e1000989. http://dx.doi.org/10.1371/journal.pcbi.1000989.
35. **Weickert MJ, Adhya S.** 1992. A family of bacterial regulators homologous to Gal and Lac repressors. J. Biol. Chem. **267:**15869–15874.
36. **Sá-Nogueira I, Ramos SS.** 1997. Cloning, functional analysis, and transcriptional regulation of the *Bacillus subtilis araE* gene involved in L-arabinose utilization. J. Bacteriol. **179:**7705–7711.
37. **Sá-Nogueira I, Nogueira TV, Soares S, Lencastre H.** 1997. The *Bacillus subtilis* L-arabinose (*ara*) operon: nucleotide sequence, genetic organization and expression. Microbiology **143:**957–969. http://dx.doi.org/10.1099/00221287-143-3-957.
38. **Doyle ME, Brown C, Helling RWHRB.** 1972. Induction of the *ara* operon of *Escherichia coli* B-r. J. Bacteriol. **110:**56–65.
39. **Schleif R, Hess W, Finkelstein S, Ellis D.** 1973. Induction kinetics of the L-arabinose operon of *Escherichia coli*. J. Bacteriol. **115:**9–14.
40. **Procházková K, Čermáková K, Pachl P, Sieglová I, Fábry M, Otwinowski Z, Řezáčová P.** 2012. Structure of the effector-binding domain of the arabinose repressor AraR from *Bacillus subtilis*. Acta Crystallogr. D Biol. Crystallogr. **68:**176–185. http://dx.doi.org/10.1107/S090744491105414X.
41. **Swint-Kruse L, Matthews KS.** 2009. Allostery in the LacI/GalR family: variations on a theme. Curr. Opin. Microbiol. **12:**129–137. http://dx.doi.org/10.1016/j.mib.2009.01.009.
42. **Colmer JA, Hamood AN.** 1998. Characterization of *ptxS*, a Pseudomonas aeruginosa gene which interferes with the effect of the exotoxin A positive regulatory gene, *ptxR*. Mol. Genet. Genomics **258:**250–259. http://dx.doi.org/10.1007/s004380050729.
43. **Meng LM, Nygaard P.** 1990. Identification of hypoxanthine and guanine as the co-repressors for the purine regulon genes of *Escherichia coli*. Mol. Microbiol. **4:**2187–2192. http://dx.doi.org/10.1111/j.1365-2958.1990.tb00580.x.
44. **Takata G, Poonperm W, Rao D, Souda A, Nishizaki T, Morimoto K, Izumori K.** 2007. Cloning, expression, and transcription analysis of L-arabinose isomerase gene from *Mycobacterium smegmatis* SMDU. Biosci. Biotechnol. Biochem. **71:**2876–2885. http://dx.doi.org/10.1271/bbb.70177.
45. **Izumori K, Elbein KYAD.** 1976. Pentose metabolism in *Mycobacterium smegmatis*: specificity of induction of pentose isomerases. J. Bacteriol. **128:**587–591.

46. **Oehler S, Eismann ER, Krámer H, Müller-Hill B.** 1990. The three operators of the *lac* operon cooperate in repression. EMBO J. **9:**973–979.
47. **Semsey S, Geanacopoulos M, Lewis DEA, Adhya S.** 2002. Operator-bound GalR dimers close DNA loops by direct interaction: tetramerization and inducer binding. EMBO J. **21:**4349–4356. http://dx.doi.org/10.1093/emboj/cdf431.
48. **Görke B, Stülke J.** 2008. Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. Nat. Rev. Microbiol. **6:**613–624. http://dx.doi.org/10.1038/nrmicro1932.
49. **Hahn S, Hendrickson W, Schleif R.** 1986. Transcription of *Escherichia coli ara in vitro*. The cyclic AMP receptor protein requirement for P_{BAD} induction that depends on the presence and orientation of the *ara*O_2 site. J. Mol. Biol. **188:**355–367.
50. **Lobell RB, Schleif RF.** 1991. AraC-DNA looping: orientation and distance-dependent loop breaking by the cyclic AMP receptor protein. J. Mol. Biol. **218:**45–54. http://dx.doi.org/10.1016/0022-2836(91)90872-4.
51. **Wendisch VF, de Graaf AA, Sahm H, Eikmanns BJ.** 2000. Quantitative determination of metabolic fluxes during coutilization of two carbon sources: comparative analyses with *Corynebacterium glutamicum* during growth on acetate and/or glucose. J. Bacteriol. **182:**3088–3096. http://dx.doi.org/10.1128/JB.182.11.3088-3096.2000.
52. **Dominguez H, Cocaign-Bonsquet M, Lindley ND.** 1997. Simultaneous consumption of glucose and fructose from sugar mixtures during batch growth of *Corynebacterium glutamicum*. Appl. Microbiol. Biotechnol. **47:**600–603. http://dx.doi.org/10.1007/s002530050980.
53. **Stansen C, Uy D, Delaunay S, Eggeling L, Goergen J-L, Wendisch VF.** 2005. Characterization of a *Corynebacterium glutamicum* lactate utilization operon induced during temperature-triggered glutamate production. Appl. Environ. Microbiol. **71:**5920–5928. http://dx.doi.org/10.1128/AEM.71.10.5920-5928.2005.
54. **Teramoto H, Inui M, Yukawa H.** 2009. Regulation of expression of genes involved in quinate and shikimate utilization in *Corynebacterium glutamicum*. Appl. Environ. Microbiol. **75:**3461–3468. http://dx.doi.org/10.1128/AEM.00163-09.