

Catabolite Repression of the *Bacillus subtilis* FadR Regulon, Which Is Involved in Fatty Acid Catabolism[∇]

Shigeo Tojo, Takenori Satomura,† Hiroshi Matsuoka, Kazutake Hirooka, and Yasutaro Fujita*

Department of Biotechnology, Faculty of Life Science and Biotechnology, Fukuyama University,
Fukuyama, Hiroshima 729-0292, Japan

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The *Bacillus subtilis* fadR regulon involved in fatty acid degradation comprises five operons, *lcfA-fadR-fadB-etfB-etfA*, *lcfB*, *fadN-fadA-fadE*, *fadH-fadG*, and *fadF-acdA-rpoE*. Since the *lcfA-fadRB-etfBA*, *lcfB*, and *fadNAE* operons, whose gene products directly participate in the β -oxidation cycle, had been found to be probably catabolite repressed upon genome-wide transcript analysis, we performed Northern blotting, which indicated that they are clearly under CcpA-dependent catabolite repression. So, we searched for catabolite-responsive elements (*cre*'s) to which the complex of CcpA and P-Ser-HPr binds to exert catabolite repression by means of a web-based *cis*-element search in the *B. subtilis* genome using known *cre* sequences, which revealed the respective candidate *cre* sequences in the *lcfA*, *lcfB*, and *fadN* genes. DNA footprinting indicated that the complex actually interacted with these *cre*'s *in vitro*. Deletion analysis of each *cre* using the *lacZ* fusions with the respective promoter regions of the three operons with and without it, indicated that these *cre*'s are involved in the CcpA-dependent catabolite repression of the operons *in vivo*.

Fatty acids are essential components of membranes and are important sources of metabolic energy in all organisms. Thus, the fatty acid degradation and biosynthesis pathways must be switched on and off according to the availability of fatty acids to maintain membrane lipid homeostasis. A soil bacterium of low GC content, *Bacillus subtilis*, possesses two transcriptional global regulators, FadR (formerly YsiA) (14) and FapR (22), which are involved in fatty acid degradation and biosynthesis, respectively (5). FadR is antagonized by long-chain acyl-coenzyme A (CoA) (14), whereas FapR is antagonized by malonyl-CoA, a building block for fatty acid biosynthesis (22). The FadR regulon comprises five *fad* operons, *lcfA-fadR* (*ysiA-fadB* (*ysiB*)-*etfB-etfA*, *lcfB* (*yhfL*), *fadN* (*yusL*)-*fadA* (*yusK*)-*fadE* (*yusJ*), *fadH* (*ykuF*)-*fadG* (*ykuG*), and *fadF* (*ywjF*)-*acdA-rpoE*, the expression of which FadR represses (14) (Fig. 1). The first three operons are considered to be directly involved in the fatty acid β -oxidation cycle. LcfA and LcfB most likely encode long-chain fatty acid-CoA ligases, FadE likely encodes acyl-CoA dehydrogenase, FadB and FadN likely encode enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase complexes, FadA likely encodes 3-ketoacyl-CoA thiolase, and EtfA and EtfB likely encode the electron transfer flavoprotein dehydrogenase α - and β -subunits.

Growing *B. subtilis* is unable to degrade straight-chain fatty acids added to the culture medium (11). Since *B. subtilis* apparently possesses the genes involved in the β -oxidation cycle, this cycle appears to have a dispensable function under certain physiological conditions. In fact, the *lcfA-fadRB-etfBA*, *lcfB*,

and *fadNAE* operons have been reported to be induced upon glucose starvation (13). Furthermore, the *fadNAE* operon is induced at the onset of sporulation (9). This induction requires the YvbA protein involved in cannibalism by sporulating cells. These findings imply that the three operons might be under catabolite repression, as the β -oxidation genes of *Escherichia coli* are positively regulated by the cyclic AMP receptor protein/cyclic AMP system for their catabolite repression (8, 18).

In *B. subtilis*, catabolite regulation occurs on the binding of the complex of the catabolite control protein (CcpA) and the seryl-phosphorylated form of a histidine-containing protein or its homologue (P-Ser-HPr or -Crh) to catabolite-responsive elements (*cre*'s) of target operons (16); P-Ser-HPr or P-Ser-Crh was formed upon activation of HPr kinase/phosphorylase (HPrK), which is triggered by an increase in the fructose 1,6-bisphosphate concentration in the presence of carbohydrates transported by the phosphoenolpyruvate-dependent phosphotransferase system, such as Glc (3, 7, 8, 10). The complex of CcpA and P-Ser-HPr (or P-Ser-Crh) triggers the expression of several genes, including those encoding the enzymes necessary for the synthesis of major extracellular products such as acetoin (3) and acetate (19). On the other hand, this complex represses many catabolic genes and operons, mainly those involved in carbon, nitrogen, and phosphate metabolism.

We investigated in this work whether or not the respective operons constituting the catabolic regulon of FadR involved in fatty acid β -oxidation are under catabolite repression. We found that out of the five *fad* operons, three (*lcfA-fadRB-etfBA*, *lcfB*, and *fadNAE*) encoding the constituents of the β -oxidation pathway are catabolite repressed. Their catabolite repression was dependent on CcpA and mediated by the respective *cre* located in the *lcfA*, *lcfB*, or *fadN* gene.

MATERIALS AND METHODS

Bacterial strains. The *B. subtilis* strains used are listed in Table 1. Strain FU788 (the Δ *fadR::cat* mutant) was transformed with plasmid pCm::Tc (23) to

* Corresponding author. Mailing address: Department of Biotechnology, Faculty of Life Science and Biotechnology, Fukuyama University, 985 Sanzo, Higashimura-cho, Fukuyama-shi, Hiroshima 729-0292, Japan. Phone: 81 84 936 2111. Fax: 81 84 936 2023. E-mail: yfujita@bt.fubt.fukuyama-u.ac.jp.

† Present address: Department of Materials Science, Yonago National College of Technology, Yonago, Tottori 683-8506, Japan.

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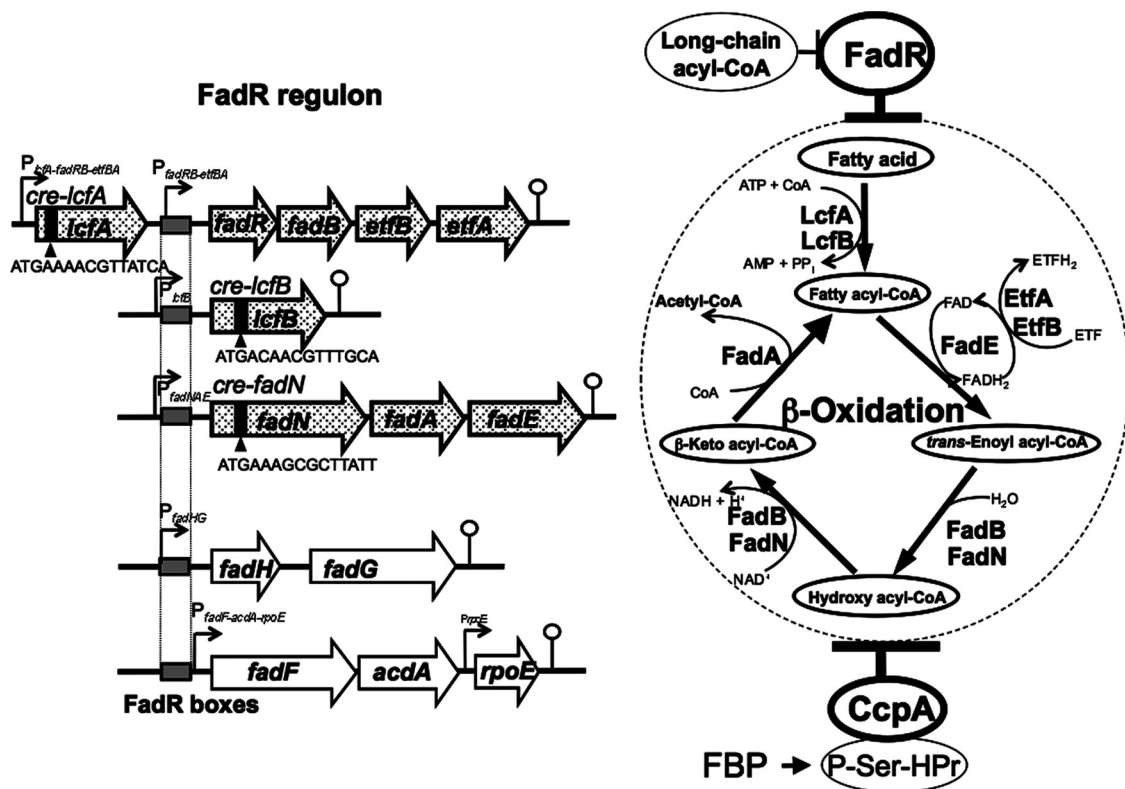


FIG. 1. The *B. subtilis* FadR regulon and its catabolite repression. The FadR regulon comprises the five operons (*lcfA-fadRB-ETFBA*, *lcfB*, *fadNAE*, *fadHG*, and *fadF-accA-rpoE*) that are involved in fatty acid β -oxidation and closely related functions (14). The *lcfA-fadRB-ETFBA* operon possesses two promoters ($P_{lcfA-fadRB-ETFBA}$ and $P_{fadRB-ETFBA}$). The FadR boxes located in the promoter regions ($P_{fadRB-ETFBA}$, P_{lcfB} , P_{fadNAE} , P_{fadHG} , and $P_{fadF-accA-rpoE}$) are indicated by gray boxes, to which FadR binds to repress transcription from them. FadR is antagonized by long-chain acyl-CoA. Three of these five operons (*lcfA-fadRB-ETFBA*, *lcfB*, and *fadNAE*) were found in this work to be under CcpA-dependent catabolite repression. The cre's (*cre-lcfA*, *cre-lcfB*, and *cre-fadN*) responsible for catabolite repression of the respective operons were located in the *lcfA*, *lcfB*, and *fadN* genes, to which the complex of CcpA and P-Ser-HPr binds. The formation of P-Ser-HPr is triggered by an increase in the concentration of fructose-1,6-bisphosphate (FBP). The gene products of the *lcfA-fadRB-ETFBA*, *lcfB*, and *fadNAE* operons, which were under CcpA-dependent catabolite repression, are directly involved in fatty acid β -oxidative degradation, and their functions in β -oxidation are indicated on the right.

change its chloramphenicol resistance to tetracycline resistance (10 μ g/ml), which yielded strain FU884 (the Δ *fadR::tet* mutant). Strain FU1084 (the Δ *fadR::tet* Δ *ccpA::neo* mutant) was constructed by the transformation of strain FU884 with DNA of strain FU402 (the *ccpA::neo* mutant) to neomycin resistant (15 μ g/ml).

To construct transcriptional fusion strains of the *lcfA-fadRB-ETFBA*, *lcfB*, and *fadNAE* promoters with *lacZ*, their promoter regions (P_{lcfA} , P_{lcfB} , and P_{fadN}) comprising nucleotides -434 to +467 and -437 (with and without the *cre* in *lcfA*), -120 to +514 and +484 (with and without the *cre* in *lcfB*), and -286 to +1130 and +1100 (with and without the *cre* in *fadN*) (+1 is the transcription initiation nucleotide; that of *lcfA-fadRB-ETFBA* was determined in this work [Fig. 2]), were amplified by PCR using primer pairs (plcA-F/plcA-Rcre and plcA-F/plcA-Rdcre, plcB-F/plcB-Rcre and plcB-F/plcB-Rdcre, and pfadN-F/pfadN-Rcre and pfadN-F/pfadN-Rdcre, respectively) (Table 2), with the DNA of strain 168 as a template. Each of the PCR products, trimmed with XbaI and BamHI (or BglII for *fadN*) digestion, was cloned into the pCRE-test2 vector (15), which had been treated with the same restriction enzymes. Correct construction was confirmed by DNA sequencing. The resultant plasmids were linearized by PstI digestion and then integrated into the *amyE* locus of strain 168 through double-crossover transformation to obtain chloramphenicol resistance, which resulted in strains FU989 [*PlcA(+cre)-lacZ*] and FU987 [*PlcA(-cre)-lacZ*], FU961 [*PlcB(+cre)-lacZ*] and FU960 [*PlcB(-cre)-lacZ*], and FU993 [*PfadN(+cre)-lacZ*] and FU991 [*PfadN(-cre)-lacZ*]. The resultant strains were then transformed with the DNA of strain FU884 (the Δ *fadR::tet* mutant) to obtain tetracycline resistance, which resulted in strains FU990 and FU988, FU964 and FU962, and FU994 and FU992, whereas strains FU989, FU961, and FU993 were also transformed with the DNA of strain FU402 (the *ccpA::neo* mutant) to obtain neomycin resistance, which resulted in strains FU1071, FU1074, and

FU1051, respectively. Doubly disrupted strains with respect to *fadR* and *ccpA* (strains FU1048, FU965, and FU1053) were obtained by transformation of strains FU988, FU964, and FU994 with DNA of strain FU402 to obtain neomycin resistance.

Preparation of the CcpA and P-Ser-HPr proteins. The CcpA protein synthesized using the cells of *E. coli* strain JM109 bearing plasmid pCCPA19 (17) was purified to over 90% homogeneity by two steps of column chromatography (DEAE-Toyopearl 650 M [Tosoh Co., Tokyo, Japan] and heparin Sepharose [GE Healthcare]). The P-Ser-HPr protein was prepared as follows. The HPr protein was synthesized using cells of *E. coli* strain BL21(DE3) bearing plasmid pET-ptsH, which had been constructed by the cloning of the *ptsH* gene (PCR amplified with a primer pair [htsH-F/htsH-R] [Table 2]) into plasmid pET22b (+) (Novagen) using the NdeI and BamHI sites, and then purified to approximately 80% homogeneity by the column chromatography of DEAE-Toyopearl, 650 M. The His₆-tagged HPr kinase was synthesized by the cells of *E. coli* strain BL21(DE3) bearing plasmid pET-hprK, which had been constructed by the cloning of the *hprK* gene (PCR amplified with a primer pair [hprK-F/hprK-R] [Table 2]) into plasmid pET16b using the NdeI and BamHI sites, and then purified to approximately 85% by column chromatography on Ni-nitrilotriacetic acid (NTA) agarose (Qiagen). Ser-46 of the HPr protein was phosphorylated *in vitro* to form P-Ser-HPr using the His₆-tagged HPr kinase under the experimental conditions described (8). After the phosphorylation reaction, the kinase was removed from the reaction mixture by passage through an Ni-NTA agarose column.

Primer extension analysis. *B. subtilis* strain FU988 cells were grown in S6 medium (4) containing 0.5% Casamino Acids (Difco) as a carbon source and 50 μ g tryptophan per ml to an optimal density at 600 nm (OD₆₀₀) of 1.0. Total RNA was extracted and purified from the cell pellet as described previously (26). To

TABLE 1. *B. subtilis* strains used in this work

Strain	Genotype	Reference or source
168	<i>trpC2</i>	1
FU788	Δ <i>fadR::cat trpC2</i>	14
FU884	Δ <i>fadR::tet trpC2</i>	This work
FU402	Δ <i>ccpA::neo trpC2</i>	24
FU1084	Δ <i>fadR::tet</i> Δ <i>ccpA::neo trpC2</i>	This work
FU989	<i>amyE::[cat PlcFA(-434/+467)(+cre)-lacZ] trpC2</i>	This work
FU987	<i>amyE::[cat PlcFA(-434/+437)(-cre)-lacZ] trpC2</i>	This work
FU1071	<i>amyE::[cat PlcFA(-434/+467)(+cre)-lacZ] Δ<i>ccpA::neo trpC2</i></i>	This work
FU990	<i>amyE::[cat PlcFA(-434/+467)(+cre)-lacZ] Δ<i>fadR::tet trpC2</i></i>	This work
FU988	<i>amyE::[cat PlcFA(-434/+437)(-cre)-lacZ] Δ<i>fadR::tet trpC2</i></i>	This work
FU1048	<i>amyE::[cat PlcFA(-434/+467)(+cre)-lacZ] Δ<i>fadR::tet</i> Δ<i>ccpA::neo trpC2</i></i>	This work
FU961	<i>amyE::[cat PlcFB(-120/+514) (+cre)-lacZ] trpC2</i>	This work
FU960	<i>amyE::[cat PlcFB(-120/+484) (-cre)-lacZ] trpC2</i>	This work
FU1074	<i>amyE::[cat PlcFB(-120/+514) (+cre)-lacZ] Δ<i>ccpA::neo trpC2</i></i>	This work
FU964	<i>amyE::[cat PlcFB(-120/+514) (+cre)-lacZ] Δ<i>fadR::tet trpC2</i></i>	This work
FU962	<i>amyE::[cat PlcFB(-120/+484) (-cre)-lacZ] Δ<i>fadR::tet trpC2</i></i>	This work
FU965	<i>amyE::[cat PlcFB(-120/+514) (+cre)-lacZ] Δ<i>fadR::tet</i> Δ<i>ccpA::neo trpC2</i></i>	This work
FU993	<i>amyE::[cat PfdN(-286/+1130)(+cre)-lacZ] trpC2</i>	This work
FU991	<i>amyE::[cat PfdN(-286/+1100)(-cre)-lacZ] trpC2</i>	This work
FU1051	<i>amyE::[cat PfdN(-286/+1130)(+cre)-lacZ] Δ<i>ccpA::neo trpC2</i></i>	This work
FU994	<i>amyE::[cat PfdN(-286/+1130)(+cre)-lacZ] Δ<i>fadR::tet trpC2</i></i>	This work
FU992	<i>amyE::[cat PfdN(-286/+1100)(-cre)-lacZ] Δ<i>fadR::tet trpC2</i></i>	This work
FU1053	<i>amyE::[cat PfdN(-286/+1130)(+cre)-lacZ] Δ<i>fadR::tet</i> Δ<i>ccpA::neo trpC2</i></i>	This work

map the 5' end of the *lcfA-fadRB-efbA* transcript, total RNA (50 μ g) was annealed to 1 pmol of primer lcfA-PE-R (Table 2), which had been 5'-end labeled with a Megalabel kit (Takara-Bio) and [γ - 32 P]ATP (MP Biomedicals), and then the primer extension reaction was conducted with ThermoScript reverse transcriptase (Invitrogen) as described previously (21). A template for the dideoxy sequencing reactions for ladder preparation was generated by PCR with the genomic DNA of strain 168 as a template and the primer pair of lcfA-PE-F/lcfA-PE-R (Table 2). An autoradiogram was obtained using a Typhoon 9400 variable image analyzer (GE Healthcare).

Northern analysis. Total RNAs were extracted and purified as described above from cells of strains 168, FU884, FU402, and FU1084, which had been grown to an OD₆₀₀ of 1 in S6 medium supplemented as described above, with and without 10 mM Glc, as described previously. RNA was electrophoresed in a glyoxal gel and then transferred to a Hybond-N membrane (GE Healthcare) (20). RNA transferred to the membrane was stained with 0.003% methylene blue to check its quality before hybridization. To prepare probes for the detection of the transcripts of *lcfA-fadRB-efbA*, *lcfB*, *fadNAE*, *fadHG*, and *fadF-actA-rpoE*, the respective *lcfA*, *lcfB*, *fadN*, *fadH*, and *fadF* fragments amplified by PCR using primer pairs (lcfA-Nn-F/lcfA-Nn-R, lcfB-Nn-F/lcfB-Nn-R, fadN-Nn-F/fadN-Nn-R, fadH-Nn-F/fadH-Nn-R, and fadF-Nn-F/fadF-Nn-R) (Table 2) and the chromosomal DNA of strain 168 as a template were labeled with a BcaBEST labeling kit (Takara-Bio, Kyoto, Japan) and [α - 32 P]-dCTP (MP Biomedicals). Hybridization and transcript detection were carried out as described previously (20).

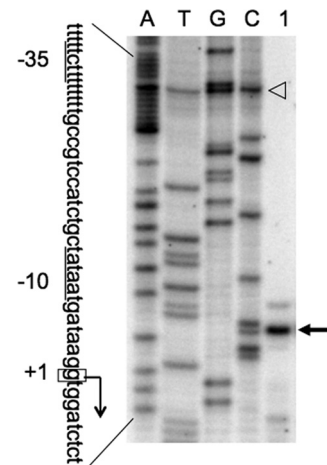


FIG. 2. Determination of the transcription start site of the *lcfA-fadRB-efbA* operon by primer extension analysis. Total RNA of strain FU988 [*PlcFA(-cre)-lacZ*; the Δ *fadR* mutant] (lane 1) was prepared and used for the reverse transcription reaction to generate runoff cDNA. Lanes A, T, G, and C contained the products of the dideoxy sequencing reactions obtained with the same primers as those used for reverse transcription; the open arrowhead indicates a false band observed in the A, T, G, and C lanes. The runoff cDNA is indicated by an arrow. The partial nucleotide sequence of the coding strand corresponding to the ladders is shown; the -35 and -10 sequences are underlined, and the transcription start site (+1) is enclosed in a box.

DNase I footprinting. DNase I footprinting experiments were performed essentially as described previously (6, 27). The *lcfA*, *lcfB*, and *fadN* probes for the footprinting of the CcpA and P-SerHPr proteins described above were synthesized by PCR with the genomic DNA of strain 168 as a template and primer pairs lcfA-FP-F/lcfA-FP-R, lcfB-FP-F/lcfB-FP-R, and fadN-FP-F/fadN-FP-R (Table 2), respectively. Prior to PCR amplification, the 5' end of only one of each primer pair was labeled as mentioned above.

TABLE 2. Oligonucleotide primers used in this work

Oligonucleotide	Sequence ^a
ptsH-FAGGAGAATGATACATATGGCAC
ptsH-RTGGATCCTTGCAATTA
hprK-FATCATATGGCAAAGGTTCCGCAACAAAGCC
hprK-RTAACGGATCCTCTCTATCTTCTGTTCC
lcfA-PE-FGGCTTCTTTTGTGGTGGCA
lcfA-PE-RCGTTGGGATCACTCGGCAAGC
lcfA-Nn-FGTCCGACAGATTTTGATAACC
lcfA-Nn-RCTGGATGGCTTCATGTTTATA
lcfB-Nn-FGCGGCGACATCTGCCGCTAAA
lcfB-Nn-RGGCAAAATATCTCTCAGTGCCC
fadN-Nn-FCCGTTTGCTGTGGAATATCAC
fadN-Nn-RGTATCGTATAAAAGATCGGGCC
fadH-Nn-FGGCGGTAATTATTACCGCGGG
fadH-Nn-RCGGATAAGGATTCAGCCATTGGCC
fadF-Nn-FCCGACGAAACGAAAGAAAGCTA
fadF-Nn-RCGTCTCATGAAGCGGGTGTCT
lcfA-FP-FTTATGGCGTCTTTGTTGCGG
lcfA-FP-RTTGTTTTTTCGTCAGCGGG
lcfB-FP-FTGAGAGCATGCATGAATCCG
lcfB-FP-RTTTTCAGTCTCTGACGCT
fadN-FP-FAAAAAGGTTGGATCGGAAGC
fadN-FP-RTTCATGGCTTGGTCAATCCG
plcfA-FTAGCTCTAGAAAAGGAAATCTTGGCGTGGC
plcfA-RGGAGGATCCGTCGACTATTGATAACGTTTTTC
plcfA-RdcreGGAGGATCTTGTCTCGGAAAAGCAAATC
plcfB-FTCTTCTAGAGGCCCTTTGATCTGACAGAAG
plcfB-RGGAGGATCCGCAATATTTTTGCAAACGTTGTCAT
plcfB-RdcreGGAGGATCTCTGACCTCTGGGTCCG
pfadN-FTCGCTCTAGATGAAAAGTGAAGCAGCCG
pfadN-RAGAAGATCTCATCCGAATAAATAAGCGCTTTC
pfadN-RdcreAGAAGATCTCTTTTGTTCGCTTTTTCGCTGTTT

^a Underlined sequences are XbaI and BamHI (or BglII) sites.

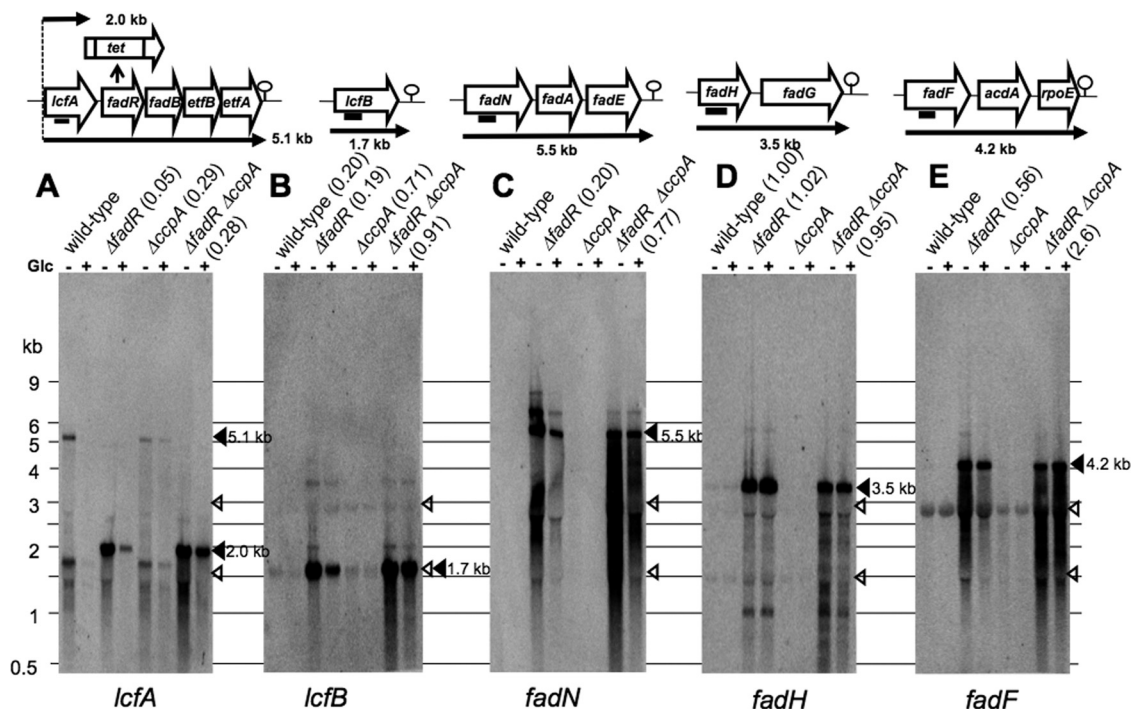


FIG. 3. Northern analysis of catabolite repression of *lcfA-fadRB-ETFBA*, *lcfB*, *fadNAE*, *fadHG*, and *fadF-accA-rpoE* transcription. Total RNAs from strains 168 (wild type), FU884 (the $\Delta fadR$ mutant), FU402 (the $\Delta ccpA$ mutant), and FU1084 (the $\Delta fadR \Delta ccpA$ mutant), which had been grown in the absence (-) and presence (+) of Glc, were subjected to Northern analysis using the *lcfA*, *lcfB*, *fadN*, *fadH*, and *fadF* probes (A, B, C, D, and E, respectively). The analysis was performed as described in the text. Each lane contained 10 μ g of total RNA. The positions of size markers are indicated on the left. The open arrowheads indicate the positions of 23 and 16 rRNAs. Solid boxes beneath the genes (top) indicate the positions of the probes. Putative transcription terminators are given downstream of the last genes of the operons. The band densities were determined with ImageQuant TL (GE Healthcare). The repression ratios (band density determined with the cells grown with Glc divided by that without Glc) are given in parentheses.

lacZ fusion analysis to monitor the promoters of *lcfA-fadRB-ETFBA*, *lcfB*, and *fadNAE*. *B. subtilis* cells were inoculated into 50 ml of S6 medium (4) supplemented as described above, with and without 10 mM Glc, to give an OD₆₀₀ of 0.1. During cell growth at 37°C with shaking, 1-ml aliquots of the culture were withdrawn at 1-h intervals, and the β -galactosidase (β -Gal) activity was measured spectrophotometrically as described previously (25).

RESULTS AND DISCUSSION

Catabolite repression of the FadR regulon. The *B. subtilis* *fadR* regulon involved in fatty acid degradation comprises five operons, *lcfA-fadRB-ETFBA*, *lcfB*, *fadNAE*, *fadHG*, and *fadF-accA-rpoE* (14) (Fig. 1). Although the transcription initiation nucleotides of *lcfB*, *fadNAE*, and *fadF-accA-rpoE* were determined previously (14), that of *lcfA-fadRB-ETFBA* was determined in this work to be a guanine at the 31st base upstream of the translation initiation base (Fig. 2). The presumed -10 sequence of the *lcfA-fadRB-ETFBA* promoter (TATAAT) perfectly matched the consensus sequence of σ^A -recognized promoters, but the -35 sequence (TTCTTT) was very different from the consensus sequence.

Out of these operons, *lcfA-fadRB-ETFBA*, *lcfB*, and *fadNAE* were found to be induced upon glucose starvation after glucose was exhausted from minimal medium by means of a DNA microarray experiment (13), which was consistent with the DNA microarray results for glucose repression in the cells logarithmically grown in nutrient sporulation medium, except for in the case of *lcfB*, whose expression was not properly

detected for an unknown reason (26) (<http://www.genome.ad.jp/kegg/expression>). In order to confirm these genome-wide profiling results, we performed Northern analysis of glucose repression of the above five operons belonging to the FadR regulon (Fig. 3). Total RNAs were prepared from cells of strains 168 (the wild type), FU884 (the $\Delta fadR$ mutant), FU402 (the $\Delta ccpA$ mutant), and FU1084 (the $\Delta fadR \Delta ccpA$ mutant), which had been mid-logarithmically grown in the absence (-) and presence (+) of Glc. Figure 3A, B, C, D, and E show Northern blotting of the *fad* transcripts with the ³²P-labeled probes of the PCR products of parts of the first genes of these operons (*lcfA*, *lcfB*, *fadN*, *fadH*, and *fadF*). The full-length 5.1-kb transcript of *lcfA-fadRB-ETFBA* was too severely repressed by Glc to be visible in the wild-type *fadR*⁺ background (Fig. 3A). However, the 5.1-kb transcript was visible in the $\Delta ccpA$ background even in the presence of Glc, so its synthesis was found to be repressed by Glc, with a repression ratio (band density for lane with Glc [+] divided by that without Glc [-]) of 0.29. This 5.1-kb transcript was truncated to 2.0 kb due to the presence of a transcription terminator in the 5' region of the *cat* cassette that remained after the insertion of the tetracycline resistance gene (*tet*) into the *cat* gene through double-crossover transformation (23), which was observed in the $\Delta fadR$ background; the 2.0-kb transcript had also been observed with strain FU788 (the $\Delta fadR::cat$ mutant) previously (14). This 2.0-kb transcript was repressed by Glc with a repres-

sion ratio of 0.05 in the wild-type background. However, the 2.0-kb transcript was well derepressed, with a ratio of 0.28 in the $\Delta ccpA$ background (this ratio is comparable to the 0.29 mentioned above for the 5.1-kb transcript in the $\Delta ccpA$ background). This also indicated that the 2.0- and 5.1-kb transcripts were still significantly repressed by Glc even in the absence of CcpA. These results indicated that the transcription from the *lcfA-fadRB-ETFBA* promoter is under CcpA-dependent catabolite repression as well as under significant but unknown CcpA-independent catabolite repression.

The 2.0-transcript of *lcfA* was considered to be induced by FadR inactivation if the band densities of the 2.0- and 5.1-kb transcripts were compared (Fig. 3A), nevertheless there was found to be no FadR box regulating the *lcfA-fadRB-ETFBA* promoter preceding *lcfA* (14). This might be consistent with the fact that the -35 sequence (TTCTTT) of this promoter is very different from the consensus sequence recognized by σ^A , implying the presence of an unknown transcriptional activator of this promoter that might be induced by FadR inactivation. This transcription activation system might possibly be under an unknown CcpA-independent catabolite repression.

The *lcfB* transcript of 1.7 kb was repressed by Glc in the wild-type and $\Delta fadR$ backgrounds (ratios of 0.20 and 0.19), respectively (Fig. 3B). These ratios were largely decreased in the *ccpA* background (ratios of 0.71 and 0.91), indicating that the *lcfB* expression is under CcpA-dependent catabolite repression. The full-length 5.5-kb transcript of the *fadNAE* operon was detected only in the $\Delta fadR$ background due to its strong repression by FadR (Fig. 3C). The 5.5-kb transcript was repressed by Glc (ratio = 0.20), whereas it was well derepressed in the $\Delta fadR \Delta ccpA$ background (ratio = 0.77). This finding indicates that the synthesis of the *fadNAE* transcript is under CcpA-dependent catabolite repression. The *fadHG* and *fadF-acdA-rpoE* operons were considered to be catabolite resistant, as judged from the above genome-wide mRNA profiling. Figure 3D shows that the *fadHG* operon is completely resistant to glucose repression not only in the wild type (ratio = 1.00) but also in the $\Delta fadR$ (ratio = 1.02) background. Figure 3E shows that the *fadF-acdA-rpoE* operon is slightly but significantly under catabolite repression in the $\Delta fadR$ background (ratio = 0.56). Unexpectedly, this operon was rather induced by Glc upon the introduction of $\Delta ccpA$ (ratio = 2.60). This finding suggests that this operon might be very weakly subject to CcpA-dependent catabolite repression.

The above Northern blotting of catabolite repression of the FadR regulon clearly indicated in the $\Delta fadR$ genetic background that the synthesis of the transcripts of *lcfA-fadRB-ETFBA*, *lcfB*, and *fadNAE* were repressed by Glc more than 5-fold, whereas that of *fadHG* and *fadF-acdA-rpoE* was hardly repressed (Fig. 3). The catabolite repression of *lcfA-fadRB-ETFBA*, *lcfB*, and *fadNAE* was mediated by CcpA, whereas that of *lcfA-fadRB-ETFBA* was only partially relieved by CcpA inactivation. As shown in Fig. 1, the gene products of the *lcfA-fadRB-ETFBA*, *lcfB*, and *fadNAE* operons except for FadR (LcfA, LcfB, FadB, FadN, FadA, FadE, EtfA, and EtfB) are directly involved in fatty acid β -oxidation, the synthesis of which was clearly under CcpA-dependent catabolite repression. This implies that the production of acetyl-CoA as an energy supplier is not needed any more if Glc is present in the medium, even if the β -oxidation cycle functions only under

certain specific physiological conditions, such as glucose starvation (13) and even if it does not degrade straight-chain fatty acids to support the growth of *B. subtilis* as a sole carbon source (11).

Analysis of the *cre* sequences involved in CcpA-dependent catabolite repression of the FadR regulon. Since *lcfA-fadRB-ETFBA*, *lcfB*, and *fadNAE* are under CcpA-dependent catabolite repression, we searched for *cre* sequences (3, 16) located in these operons, to which the complex of CcpA and P-Ser-HPr binds to exert catabolite repression (3, 7). A web-based *cis*-element search of the *B. subtilis* genome (<http://dbtbs.hgc.jp/motiflocationsearch.html>) using 50 known *cre* sequences (3) revealed the respective candidate *cre* sequences (ATGAAAA CGTTATCA, ATGACAACGTTTGCA, and ATGAAAGCG CTTATT) in the *lcfA*, *lcfB*, and *fadN* genes (*cre-lcfA*, *cre-lcfB*, and *cre-fadN*), which comprise nucleotides +443 and +457, +490 and +504, and +1106 and +1020, respectively. We examined, by DNase I footprinting, whether or not the complex of CcpA and P-Ser-HPr is specifically bound. P-Ser-HPr was prepared by *in vitro* phosphorylation of HPr using His₆-tagged HPr kinase as described above. A specific interaction of the complex of CcpA and P-Ser-HPr with each of the candidate *lcfA*, *lcfB*, and *fadN* *cre*'s on the coding and noncoding strands was clearly observed, as shown in Fig. 4, in which the regions protected against DNase I are indicated by thick bars. As shown at the bottom of Fig. 4, each *cre* sequence is completely included in the respective protected regions of the coding and noncoding strands. Thus, the candidate *cre*'s were highly likely involved in catabolite repression of the *lcfA-fadRB-ETFBA*, *lcfB*, and *fadNAE* operons.

There exists another promoter for the transcription of *fadRB-ETFBA* (Fig. 1) (14). However, we found no candidate *cre* in the promoter and transcribed region of *fadRB-ETFBA* by a web-based *cis*-element search of the *B. subtilis* genome mentioned above. Actually, the 2.0-kb transcript of *lcfA* was derepressed with a glucose repression ratio of 0.28 in the $\Delta ccpA$ background, which is comparable to 0.29 for a 5.1-kb transcript in the $\Delta ccpA$ background (Fig. 3A). This coincides with the *lcfA-fadRB-ETFBA* region containing only one *cre* in *lcfA*. Hence, it was considered that the transcription of *fadRB-ETFBA* could not be under the CcpA-dependent catabolite repression.

Deletion analysis of each of the *cre*'s in the *lcfA*, *lcfB* and *fadN* genes. To examine whether or not *cre-lcfA*, *cre-lcfB*, and *cre-fadN* are each actually involved in catabolite repression *in vivo*, the promoter regions of *lcfA-fadRB-ETFBA*, *lcfB*, and *fadNAE* with and without them [$P_{lcfA}(+cre)$ and $P_{lcfA}(-cre)$; $P_{lcfB}(+cre)$ and $P_{lcfB}(-cre)$; and $P_{fadN}(+cre)$ and $P_{fadN}(-cre)$] were fused with *lacZ*, and the fusions were integrated into the *amyE* loci of the wild-type, $\Delta ccpA$, $\Delta fadR$, and $\Delta fadR \Delta ccpA$ strains, respectively. First, cells of the $P_{lcfA}(+cre)$ -*lacZ* strains, i.e., FU989 (the wild type), FU1071 (the $\Delta ccpA$ mutant), FU990 (the $\Delta fadR$ mutant), and FU1048 (the $\Delta ccpA \Delta fadR$ mutant) (Fig. 5A, columns 1, 2, 5 to 8, 11, and 12, respectively), and $P_{lcfA}(-cre)$ -*lacZ* strains, FU987 (the wild type) and FU988 (the $\Delta fadR$ mutant) (Fig. 5A, columns 3, 4, 9, and 10), were grown in the absence (-) and presence (+) of Glc, and then the β -Gal activities in the cells were determined at an OD₆₀₀ of 1. The activities in the wild-type and $\Delta fadR$ mutant cells grown without Glc (Fig. 5A, columns 1 and 7) are higher than those with Glc (Fig. 5A, columns 2 and 8). β -Gal activity ratios

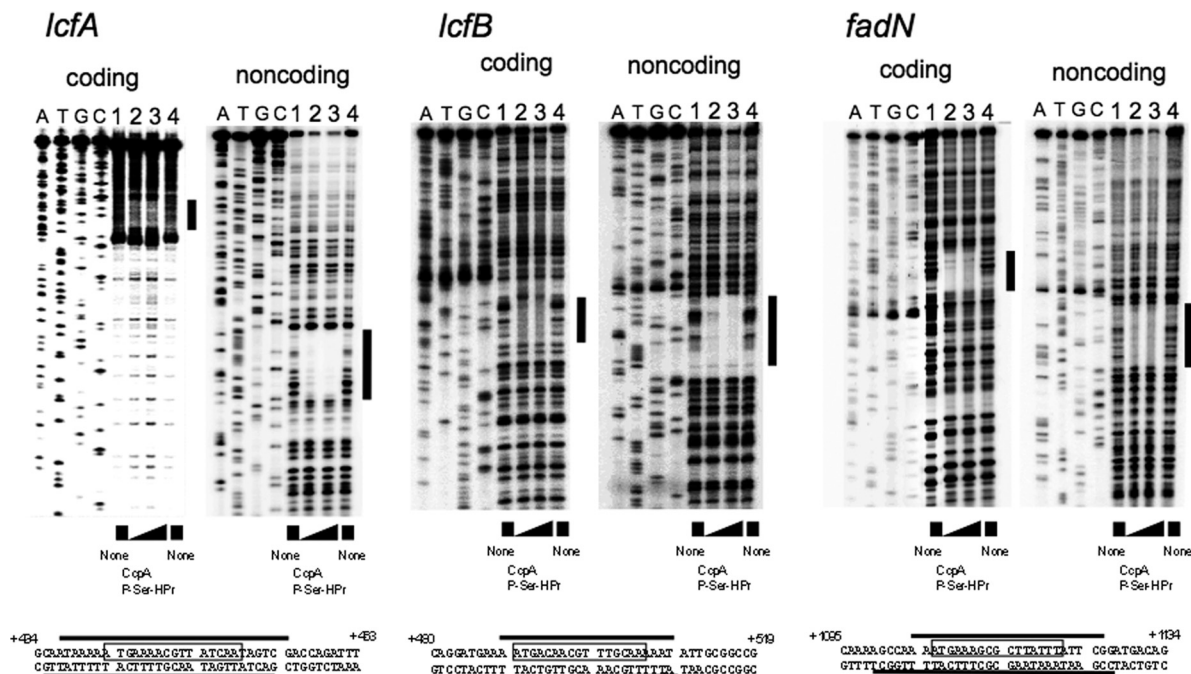


FIG. 4. DNase I footprinting of the complex of CcpA and P-Ser-HPr with the *cre-lcfA*, *cre-lcfB*, and *cre-fadN* sequences. Analysis using the 238-bp *lcfA*, 211-bp *lcfB*, and 225-bp *fadN* probes was performed as described in the text. The left and right ladders are DNA footprints of the 5'-end-labeled coding and noncoding strands, respectively, of the *lcfA*, *lcfB*, and *fadN* probes. Lanes 1 to 4 contained 0.05 pmol of the ³²P-labeled probe DNA in the reaction mixture (50 μl). Lanes 1 and 4 contained no protein. Lane 2 contained 6 μM CcpA and 8.6 μM P-Ser-HPr, and lane 3 contained 12 μM CcpA and 16.6 μM P-Ser-HPr. The assay mixture containing CcpA and P-Ser-HPr was first incubated for 10 min at 30°C before addition of the DNA probe. Lanes A, T, G, and C contained the products of the corresponding dideoxy sequencing reactions performed with the same primers as those used for probe preparation. The protected regions in ladders are indicated by black vertical bars. The nucleotide sequences of the protected regions of the coding and noncoding strands of each probe (thick horizontal lines) are shown beneath the footprints of the respective probes; the *cre* sequences are boxed.

(+Glc/−Glc) of columns 2/1 and 8/7 in Fig. 5A are 0.40 and 0.40, respectively; the values (Fig. 5A, columns 1 and 2) are quite low due to repression by FadR, exhibiting relatively high standard deviations. However, the activities in the P_{lcfA}(−*cre*)-*lacZ* and P_{lcfA}(+*cre*)-*lacZ* Δ*ccpA* mutant cells, which were grown with Glc, were very close to those without Glc (Fig. 5A, columns 4/3 and 10/9, 0.75 and 0.84; and columns 6/5 and 12/11, 0.57 and 0.85, respectively); the *cre* or CcpA dependency of the catabolite repression of P_{lcfA-fadRB-*etfBA*} is clearly observed in its well-derepressed state of the Δ*fadR* mutant (Fig. 5A, columns 9 to 12). These results indicate clearly that *cre-lcfA* is actually involved in CcpA-dependent catabolite repression of *lcfA-fadRB-*etfBA** *in vivo*. The CcpA-independent catabolite repression of P_{lcfA-fadRB-*etfBA*} detected in Northern blotting (Fig. 3A) was observed somewhat in the *lacZ* fusion experiments (Fig. 5A).

Similarly, the β-Gal activities in the P_{lcfB}(+*cre*)-*lacZ* and P_{lcfB}(−*cre*)-*lacZ* cells were determined (Fig. 5B, columns 1 to 12). *cre-lcfB* in the wild-type and Δ*fadR* mutant cells caused catabolite repression of P_{lcfB} (Fig. 5B, columns 2/1 and 8/7, 0.31 and 0.29), respectively; this repression was not observed in the P_{lcfB}(−*cre*)-*lacZ* strains (Fig. 5B, columns 4/3 and 10/9, 0.69 and 0.97, respectively) or in the P_{lcfB}(+*cre*)-*lacZ* Δ*ccpA* strains (Fig. 5B, columns 6/5 and 12/11, 0.86 and 0.93, respectively). The results clearly indicate that the *cre-lcfB* is actually involved in CcpA-dependent catabolite repression of *lcfB in vivo*.

The β-Gal activities in the wild-type and Δ*ccpA*, Δ*fadR*, and Δ*ccpA* Δ*fadR* mutant cells carrying P_{fadN}(+*cre*)- and P_{fadN}(−*cre*)-*lacZ*, which were grown in the absence and presence of Glc, were also determined (Fig. 5C, columns 1 to 12). The *cre-fadN* in the wild-type and Δ*fadR* mutant cells caused catabolite repression of P_{fadN} (Fig. 5C, columns 2/1 and 8/7, 0.36 and 0.19, respectively). The activities in the P_{fadN}(−*cre*)-*lacZ* and P_{fadN}(+*cre*)-*lacZ* Δ*ccpA* mutant cells were less repressed by Glc (Fig. 5C, columns 4/3 and 10/9, and 6/5 and 12/11, 0.44 and 0.59, and 0.75 and 0.55, respectively), but the ratios were significantly lower than the corresponding ratios for the P_{lcfA}- and P_{lcfB}-*lacZ* strains described above. These results suggest that CcpA-dependent catabolite repression involving the *cre-fadN* is not so strict, with the result that residual catabolite repression was observed even in the *ccpA* background. This might be possibly due to the relatively low affinity of the *cre-fadN* toward the complex of CcpA and P-Ser-HPr *in vivo* and (or) the presence of another *cre* upstream of it for more strict catabolite repression, as in the case of the *iol* operon (15). Anyway, it was obvious that the *cre-fadN* also functions in the catabolite repression of the *fadNAE* operon *in vivo*.

As reviewed previously (3), the complex of CcpA and P-Ser-HPr triggers the expression of several genes involved in the formation of acetate and acetoin, major extracellular products of *B. subtilis* grown on Glc. It also triggers the expression of an anabolic operon (*ilv-leu*) involved in the biosynthesis of

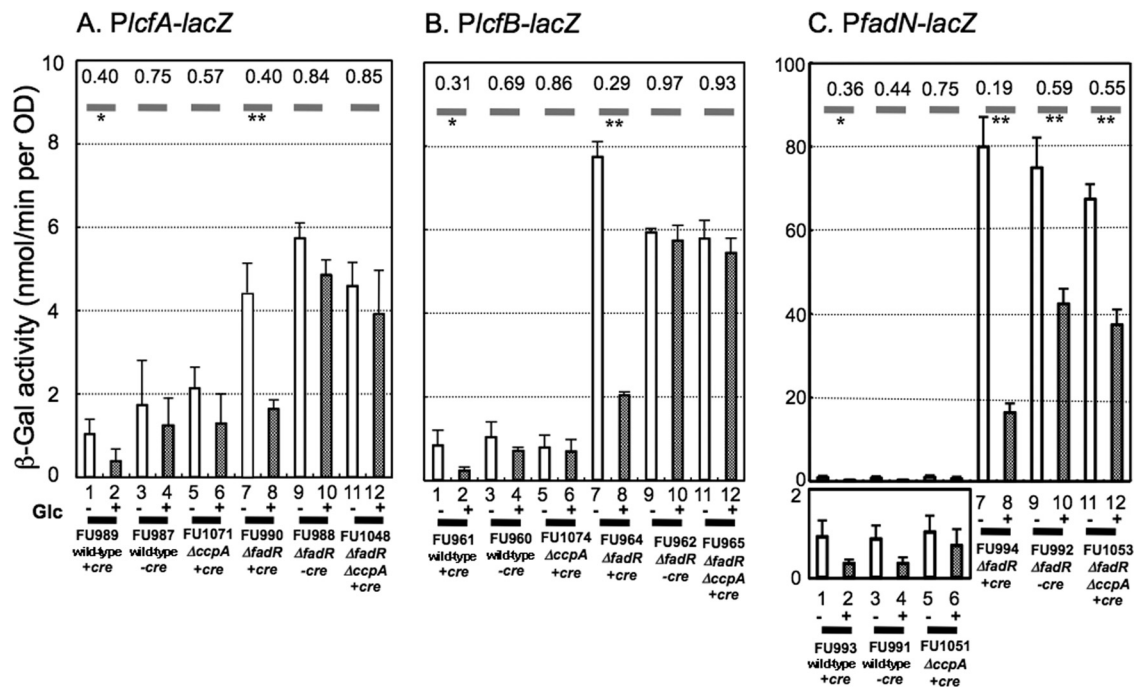


FIG. 5. Examination of *lacZ* expression under the control of each of the *lcfA-fadRB-etfBA*, *lcfB*, and *fadNAE* promoters (P_{lcfA} , P_{lcfB} , and P_{fadN}) in the $\Delta fadR$ and (or) $\Delta ccpA$ backgrounds, and deletion analysis of the *cre-lcfA*, *cre-lcfB*, and *cre-fadN* sequences. The *lcfA*, *lcfB*, and *fadN* promoter regions with and without the *cre* were placed upstream of *lacZ*, and each of the resultant *lacZ* fusions (P_{lcfA} -*lacZ*, P_{lcfB} -*lacZ*, and P_{fadN} -*lacZ* with and without the *cre*) was integrated into the *amyE* loci of the wild-type, $\Delta fadR$, $\Delta ccpA$, and $\Delta fadR$ $\Delta ccpA$ strains. (A) *lacZ* expression under the control of each of the promoters was monitored during growth with and without Glc, as described in the text, using P_{lcfA} -*lacZ* strains FU989 (wild type, +*cre*) (columns 1 and 2, -/+ Glc), FU987 (wild type, -*cre*) (3 and 4, -/+ Glc), FU1071 (the $\Delta ccpA$ mutant, +*cre*) (5 and 6, -/+ Glc), FU990 (the $\Delta fadR$ mutant, +*cre*) (7 and 8, -/+ Glc), FU988 (the $\Delta fadR$ mutant, -*cre*) (9 and 10, -/+ Glc), and FU1048 (the $\Delta fadR$ $\Delta ccpA$ mutant) (11 and 12, -/+ Glc). P_{lcfB} -*lacZ* strains (B), FU961 (wild type, +*cre*), FU960 (wild type, -*cre*), FU1074 (the $\Delta ccpA$ mutant, +*cre*), FU964 (the $\Delta fadR$ mutant, +*cre*), FU962 (the $\Delta fadR$ mutant, -*cre*), and FU965 (the $\Delta fadR$ $\Delta ccpA$ mutant), and P_{fadN} -*lacZ* strains (C), FU993 (wild type, +*cre*), FU991 (wild type, -*cre*), FU1051 (the $\Delta ccpA$ mutant, +*cre*), FU994 (the $\Delta fadR$ mutant, +*cre*), FU992 (the $\Delta fadR$ mutant, -*cre*), and FU1053 (the $\Delta fadR$ $\Delta ccpA$ mutant), were also used; the column assignments for P_{lcfB} -*lacZ* and P_{fadN} -*lacZ* are the same as those for the P_{lcfA} -*lacZ* strains. The average β -Gal activities at an OD_{600} of 1, with standard deviations, which were obtained in two independent *lacZ* monitoring experiments, are shown. In the case that the LacZ activity was less than 2 nmol/min per OD, the maximal activity in the y axis is also set to 2 nmol/min per OD. The glucose-repression ratios of each strain are shown on bold gray bars. Significant differences between the average β -Gal activities are shown by ** ($P < 0.05$) and * ($0.05 < P < 0.15$) (Student's *t* test).

branched-chain amino acids (24). On the other hand, this complex represses many genes and operons, including an entrance gene for the tricarboxylic acid (TCA) cycle (*citZ*) (12), several transporter genes for TCA cycle intermediates, some respiration genes, and many catabolic and anabolic genes involved in carbon, nitrogen, and phosphate metabolism, as well as ones for certain extracellular enzymes and secondary metabolites. It was demonstrated in this work that the complex also repressed the catabolic operons involved in the fatty acid β -oxidation cycle (*lcfA-fadRB-etfBA*, *lcfB*, and *fadNAE*).

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