Organization and Function of the YsiA Regulon of Bacillus subtilis Involved in Fatty Acid Degradation*

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The organization and function of the Bacillus subtilis YsiA regulon involved in fatty acid degradation were investigated. Northern and primer extension analyses indicated that this regulon comprises five operons, i.e. lcfA-ysiA-B-efTB-A, ykuF-G, yhfL, yusM-L-K-J, and ywjF-acdA-rpoE. YusJ and AcdA, YsiB and YusL, and YusK presumably encode acyl-CoA dehydrogenases, 3-hydroxy-CoA dehydrogenase/enoyl-CoA hydratase complexes, and acetyl-CoA C-acyltransferase, respectively, which are directly involved in the fatty acid β-oxidation cycle. In addition, LcfA and YhfL are likely to encode long chain acyl-CoA ligases. On gel retardation and footprinting analyses involving the purified YsiA protein, we identified cis-sequences for YsiA binding (YsiA boxes) in the promoter regions upstream of ysiA, ykuF, yusL, yhfL, and ywjF, the equilibrium dissociation constants (Kd) for YsiA binding being 20, 21, 37, 43, and 65 nM, respectively. YsiA binding was specifically inhibited by long chain acyl-CoAs with 14–20 carbon atoms, acyl-CoAs with 18 carbon atoms being more effective; out of long chain acyl-CoAs tested, monounsaturated oleoyl-CoA, and branched chain 12-methyltetradecanoyl-CoA were most effective. These in vitro findings were supported by the in vivo observation that the knock-out of acyl-CoA dehydrogenation through yusL, etfA, or etfB disruption resulted in YsiA inactivation, probably because of the accumulation of long chain acyl-CoAs in the cells. Furthermore, the disruption of yusL, yusK, ysfA, etfA, etfB, or ykuG affected the utilization of palmitic acid, a representative long chain fatty acid. Based on this work, ysiA, ysiB, ykuF, ykuG, yhfL, yusM, yusL, yusK, ysfL, and ywjF can be renamed fadR, fadB, fadH, fadG, lcfB, fadM, fadN, fadA, fadE, and fadF.

In all organisms fatty acids are essential components of membranes and are important sources of metabolic energy. Thus, fatty acid degradation and biosynthesis pathways must be switched on and off based on the availability of fatty acids. The regulation of these pathways has been mainly studied in a model prokaryote, Escherichia coli (1). E. coli produces straight chain and unsaturated fatty acids, and E. coli FabH selectively uses acetyl-CoA to initiate the fatty acid biosynthesis pathway (2, 3). In contrast, Bacillus subtilis mainly produces branched chain fatty acids and possesses two FabH isoenzymes (named FabHA and FabHB) that differ from the E. coli enzyme in that they are selective for branched chain acyl-CoAs (4). The second FabF-FabB class of condensing enzymes is responsible for the subsequent rounds of fatty acid elongation in the pathway (5). In B. subtilis, the FabF protein is the sole condensing enzyme able to carry out the subsequent elongation reactions in fatty acid synthesis (6). Fatty acids that are intracellularly formed or extracellularly supplied are degraded through β-oxidation when the cells are starved of carbon sources.

In E. coli, the transcription factor FadR functions as a coordinate switch that negatively regulates the machinery required for fatty acid β-oxidative degradation (fad) and positively regulates the key enzymes (3-ketoacyl-acyl carrier protein dehydratase (FabA) and 3-ketoacyl-acyl carrier protein synthase I (FabB)) in unsaturated fatty acid biosynthesis (5, 7–9). The fab genes are fadL encoding a fatty acid transporter, and fadD, fadE, fadF, fadA, and fadH involved in fatty acid β-oxidation encoding fatty acid-CoA ligase, acyl-CoA dehydrogenase, 3-hydroxyacyl-CoA dehydrogenase/enoyl-CoA hydratase complex, acetyl-CoA C-acyltransferase, and 2,4-dienoyl-CoA reductase, respectively (8, 10). Besides, FadR activates expression of the iclR gene, which encodes the negative regulator of the glyoxylate shunt enzymes, and represses the universal stress protein gene (uspA). The expression of the FadR regulon members is regulated in a coordinated manner in the presence of long chain acyl-CoAs, which antagonize FadR as to its operator binding (11).

In B. subtilis, a central mediator such as FadR that acts to balance the anabolic and catabolic fatty acid pathways has not been reported so far. Instead, a transcription regulatory protein for FapR was recently described (12) that controls the expression of many fap regulon members involved in fatty acid and phospholipid metabolism, including fapHA and fapHB, and fabF encoding the enzymes condensing branched chain acyl-CoAs with malonyl-acetyl carrier protein and subsequent chain elongation, respectively. The FapR protein is controlled by the cellular pool of malonyl-CoA, which senses the status of fatty acid biosynthesis and adjusts the expression of the fap regulon members accordingly. B. subtilis possesses several paralogous genes that are most likely involved in the β-oxidation of fatty acids, such as lcfA, yhfL, yhfT, ysiB, yusLKJ, acdA, yhfS, and mmgABC (13–16) (see Fig. 1), yet mmgABC is an ω-dependent...
oponon (13). Nevertheless, a transcription factor such as FadR that functions as a coordinate switch that regulates fatty acid degradation negatively and fatty acid biosynthesis positively has not been described so far.

In the framework of the Japan Functional Analysis Network for B. subtilis (bacillus.genome.jp), we have performed comprehensive DNA microarray analysis of hundreds of DNA-binding transcription regulatory proteins. DNA microarray analysis involving the wild type strain and a disruptant as to ysiA, encoding one of the helix-turn-helix transcription regulatory proteins, indicated that the YsiA protein negatively regulates more than 10 genes, the majority of which most likely participate in fatty acid β-oxidation, which presumably plays a central regulatory role in fatty acid degradation. In this communication, we describe the organization and function of the YsiA regulon comprising the lcfA-ysiA-B-etfB-A, ykuF-G, yhfL, yusM-L-K-J, and ywjF-acdA-rpoE operons. YsiA represses the transcription of the genes, except lcfA and yusM, belonging to these operons through its binding to their YsiA boxes. The disruption of etfA, etfB, ykuG, yusL, yusK, and yusJ affected cell growth on fatty acid as the sole carbon source. The in vitro as well as in vivo experiments suggested that long chain acyl-CoAs are most likely inducers (or ligands) that antagonize YsiA as to its binding to YsiA boxes.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Their Construction—The B. subtilis strains used in this work are listed in Table 1. Strain FU788, provided by K. Kobayashi (Nara Institute of Science and Technology, Japan), was constructed in the framework of the Japan Functional Analysis Network for B. subtilis (JAFAN; bacillus.genome.jp) through the transformation of strain 168 with a PCR product prepared by means of long flanking homology PCR (19) (the first run of PCR with primer pairs of ysiA-F1/ysiA-R1, ysiA-F2/ysiA-R2, and pUC-F-cat)/pUC-R-cat (Table 2), using strain 168 DNA and the cat gene (20) as templates, and the second PCR with ysiA-F1/ysiA-R2 (Table 2) and the above three PCR products as templates, respectively. Strains YKUFd and YHFLd were constructed as described previously (21), using primer pairs YKUFd-H/YKUFd-B, and YHFLd-H/YHFLd-B (Table 2), respectively.

DNA Microarray and Northern Analyses—DNA microarray analysis was performed similarly to as described previously (22–24). RNA samples were prepared from the cells of strains 168 (wild type) and FU788 (ysiA::cat) that had been grown in LB medium (25) and harvested in the middle of the logarithmic growth at A600 = 0.5.

For Northern analysis, the above RNA samples were electrophoresed in a glyoxal gel and then transferred to a Hybond-N membrane (GE Healthcare) (25). To prepare the labeled probes for the detection of transcripts carrying ykuF, G, yhfL, yusL, K, J, ywjF, acdA, rpoE, lefA, ysiA, and B, the products respectively amplified by PCR using primer pairs (Nykuf-F/Nykuf-R, Nykuf-G/Nykuf-R, Nyhfl-F/Nyhfl-R, NyusL-F/NyusL-R, NyusK-F/NyusK-R, NyusJ-F/NyusJ-R, Nywjj-F/Nywjj-R, NacdA-F/NacdA-R, NrpoE-F/NrpoE-R, Nlcfa-F/Nlcfa-R, NysiA-F/NysiA-R, and NysiB-F/NysiB-R; Table 2) and chromosomal DNA of strain 168 as a template were labeled with a BcaBEST labeling kit (Takara Bio, Japan) and [α-32P]dCTP (GE Healthcare). Hybridization was carried out as described previously (25). The transcripts were detected with imaging plates of Bioimaging Analyzer (Fuji Photo Film Co., Ltd).

Primer Extension Analysis—Primer extension analysis was performed as described previously (26). Total RNA was extracted and purified from cell pellets as described above. Reverse transcription was initiated from the EysA-R, Eykuf-R, Eyhfl-R, EyusL-R, and Eywjj-F-R primers, corresponding to nucleotides +122 to +141, +146 to +165, +102 to +121, +141 to +160, and +111 to +131 (+1 is the transcription initiation base for each of the YsiA-regulated promoters, which was identified in this work), respectively (Table 2); they had been labeled at their 5’ end by use of a Megalabel kit (Takara Bio) and [γ-32P]ATP (GE Healthcare). Templates for the dideoxy sequencing reactions for ladder preparation starting from the same end-labeled primer were prepared by PCR using the respective primer pairs (Table 2; EysiA-F/EysiA-R, Eykuf-F/Eykuf-R, Eyhfl-F/Eyhfl-R, EyusL-F/EysL-R, and Eywjj-F/Eywjj-R) and DNA from strain 168 as a template.

Production and Purification of YsiA—To produce the YsiA protein in E. coli, the ysiA region was amplified by PCR using a primer pair (YsiA-N/YsiA-H, Table 2) and DNA of strain 168 as a template. After digestion with Ndel and HindIII of the PCR product and vector pET-22b (+) (Novagen), the resulting fragments were ligated and then used for the transformation of E. coli BL21 (DE3). Correct cloning of ysisA in plasmid pET-22b (+)-YsiA was confirmed by nucleotide sequencing.

The YsiA protein was overexpressed in E. coli BL21 (DE3) bearing pET-22b (+)-YsiA by the addition of isopropyl-β-d-thiogalactopyranoside (IPTG)2 to the medium at 1 mm. The cells were harvested, washed with 50 mm Tris-Cl buffer (pH 8.0) two times, and then suspended in 50 mm Tris-Cl buffer (pH 8.0) containing 1% (w/v) glycerol. The cells were disrupted by sonication, and a supernatant was obtained by centrifugation (10,000 × g, 10 min). The YsiA protein was purified with an anion exchange column chromatography (TOYO PEARL (DEAE-650M) (TOSOH Corp., Japan)) to near homogeneity.

2 The abbreviations used are: IPTG, isopropyl-β-d-thiogalactopyranoside; β-Gal, β-galactosidase.
The purified YsiA protein was subjected to gel filtration column chromatography on Sephacryl S-200 HR (GE Healthcare) using 50 mM Tris-Cl buffer (pH 8.0) containing 1% (w/v) glycerol and 100 mM NaCl to determine its molecular mass.

Gel Retardation and DNase I Footprinting Experiments—Gel retardation and DNase I footprinting experiments were performed as described previously (27). For gel retardation analysis, the labeled probe DNAs carrying cis-elements for YsiA binding (YsiA boxes) of the ysiA, yhfL, ykuF, yusL, and ywjF promoters were PCR products amplified from DNA of strain 168 using primer pairs (EysiA-F/EysiA-R, EyhfL-F/EyhfL-R, PykuF-F/PykuF-R, PyusL-F/PyusL-R, and EywjF-F/EywjF-R; Table 2) in the presence of [α-32P]dCTP (GE Healthcare), respectively. For DNase I footprinting, the probe DNAs were prepared by PCR amplification using the same respective primer pairs, either of the primers having been labeled at the 5'-terminus with a Megalabel kit and [γ-32P]ATP (GE Healthcare).

Chemical Synthesis of Branched Long Chain Acyl-CoAs—12-Metyltetradecanoyl-CoA and 13-metyltetradecanoyl-CoA were chemically synthesized from 12-metyltetradecanoic acid and 13-metyltetradecanoic acid (Larodan Fine Chemicals AB) by the method of Seubert (28) with minor modifications. The branched long chain fatty acids were reacted with oxalyl chloride to form acyl chlorides. The acyl chlorides were converted with CoA to acyl-CoAs. The amounts of the formed acyl-CoAs were determined by using gas chromatography.

### TABLE 2
Primer sequences used in this study

| Primer | Sequence† |
|--------|-----------|
| ysiA disruption | GCTTGGCCGCTCCGATAGCC |
| ysiA-F2 | C07TCTGACTGCGAGAAACTATGTGTTGATCGGAAATTCAC |
| ysiA-R1 | GTAACCTCAGCTCAGGCAATGTTGCTT |
| ysiA-R2 | TACGCGCGACTGCGGACAGCC |
| pUC-F(cat) | GATATTGACCGGATATAAC |
| pUC-R(cat) | GCTTGGCCGCTCGATAGCC |
| YKUfd and YHFLd construction | GCCGAGACCTTAAAGCGCATGGGATAAGCG |
| YKUfd-H | GCCGAGACCTTAAAGCGCATGGGATAAGCG |
| YKUfd-L | GCCGAGACCTTAAAGCGCATGGGATAAGCG |
| YHFLd-H | GCCGAGACCTTAAAGCGCATGGGATAAGCG |
| YHFLd-L | GCCGAGACCTTAAAGCGCATGGGATAAGCG |
| ysiA cloning | GGAAGGAGGAACTGAGCAGAGAAGC |
| YsiA-N | GCCAAGCTTTCTCAACGCAATGAGAGC |
| YsiA-H | GCCAAGCTTTCTCAACGCAATGAGAGC |

† Underlined sequences are the sites of restriction enzymes used for strain construction.

The purified YsiA protein was subjected to gel filtration column chromatography on Sephacryl S-200 HR (GE Healthcare) using 50 mM Tris-Cl buffer (pH 8.0) containing 1% (w/v) glycerol and 100 mM NaCl to determine its molecular mass.

Gel retardation and DNase I Footprinting Experiments—Gel retardation and DNase I footprinting experiments were performed as described previously (27). For gel retardation analysis, the labeled probe DNAs carrying cis-elements for YsiA binding (YsiA boxes) of the ysiA, yhfL, ykuF, yusL, and ywjF promoters were PCR products amplified from DNA of strain 168 using primer pairs (EysiA-F/EysiA-R, EyhfL-F/EyhfL-R, PykuF-F/PykuF-R, PyusL-F/PyusL-R, and EywjF-F/EywjF-R; Table 2) in the presence of [α-32P]dCTP (GE Healthcare), respectively. For DNase I footprinting, the probe DNAs were prepared by PCR amplification using the same respective primer pairs, either of the primers having been labeled at the 5’ terminus with a Megalabel kit and [γ-32P]ATP (GE Healthcare).

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Cell Growth and β-Galactosidase (β-Gal) Assay—Cells of pMUTIN (29) disruptants of the YsiA regulon members (Table 1), were grown at 30 °C on tryptose blood agar base (Difco) plus 10 mM glucose containing erythromycin (0.3 μg/ml) overnight. The cells were inoculated into 50 ml of LB medium with and without 1 mM IPTG and then incubated at 37 °C. During growth, 1-ml aliquots of the culture were withdrawn, and the β-Gal activity in the cells was spectrophotometrically assayed, as described previously (21).

To examine the utilization of fatty acid by the pMUTIN disruptant as the sole carbon source, the cells pregrown on tryptose blood agar base plus glucose plates as described above were suspended in S6 medium (30) containing tryptophan, and then the cells (A600 × ml = 0.5) were spread on S6 solid medium containing 50 μg/ml tryptophan and 4.35 mM sodium palmitate. The cells grown on each plate at 37 °C were suspended in 5 ml of S6 medium containing tryptophan to measure A600. The β-Gal activity in the cells was assayed as described above.

RESULTS

Candidate YsiA-regulated Genes Detected on DNA Microarray Analysis—The YsiA protein is a member of the TetR family of bacterial helix-turn-helix transcriptional regulatory proteins according to Pfam (31). Within the framework of the Japan Functional Analysis Network for B. subtilis, we performed DNA microarray analysis to find candidate YsiA-regulated genes through comparison of the transcriptomes from cells of the ysiA disruptant (strain FU788) and parental wild type strain 168 grown to the logarithmic growth phase in LB medium. The analysis revealed candidate YsiA-repressed genes of ykuF, G, yusM, L, K, J, ywjF, acdA, rpoE, yhfL, lcfA, ysiB, etfA, etfB, and ysiA itself. All of these genes except yhfL are clustered in the respective orders of ykuF and G; yusM, L, K, and J; ywjF, acdA, and rpoE; and lcfA, ysiA, B, etfB, and A genes. A BLASTP sequence similarity search (17) for these gene products revealed their probable functions to be as follows (Fig. 1). The YusL and YsiB, YusK, and YusJ and AcdA proteins exhibited high sequence similarities to the 3-hydroxyacyl-CoA dehydrogenase/acyl-CoA hydratase complexes, acetyl-CoA C-acetyltransferases, and acyl-CoA dehydrogenases of many Gram-positive low GC bacteria, respectively. The LcfA and YhfL proteins exhibited high similarity to long chain fatty acid-CoA ligases. Moreover, the EtfA and EtfB proteins exhibited high sequence similarities to the α- and β-subunits of electron transfer flavoproteins, respectively. These enzymes are known to be involved in fatty acid β-oxidation. The products of the genes (LcfA, YhfL, AcdA, YusJ, YusL, YsiB, EtfA, and EtfB) could be well assigned in each of the enzymatic steps in the β-oxidation of fatty acids according to their probable functions (Fig. 1). In addition, YkuF showed significant sequence similarity to the 2,4-dienoyl-CoA reductases of Gram-positive bacteria belonging to Bacillus and related genera. The YwJF protein, a 4Fe-4S ferredoxin protein, might possibly be involved in electron transfer associated with acyl-CoA dehydrogenation, and the YkuG protein possessing a peptidoglycan-binding domain might possibly participate in fatty acid transport as described below. However, we currently cannot explain how RpoE (RNA polymerase δ-subunit) and YusM similar to proline dehydrogenases participate in fatty acid degradation.

Operon Organization of YsiA-regulated Genes—The YsiA regulon appears to be involved in fatty acid degradation. To reveal the operon organization of YsiA-repressed genes, their transcripts were analyzed by Northern blotting using the respective DNA probes for these genes and total RNA from cells of strains FU788 (ysiA::cat) and 168 grown to the logarithmic growth phase in LB medium (Figs. 2 and 3). A 3.5-kb transcript was detected with either the ykuF or ykuG probe only in the lanes of RNA derived from the ysiA disruptant, indicating that the two genes constitute an

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**FIGURE 1. Fatty acid β-oxidation pathway and the probable involvement of YsiA regulon members in it.** The YsiA regulon includes 15 genes organized into five operons (lcfA-ysiA-B-etfB-A, ykuF-G-yhfL, yusM-L-K-J, and ywjF-acdA-rpoE). A BLASTP sequence similarity search (17) of these gene products, except YwJF, YkuG, YusM, and RpoE, revealed probable functions as to the fatty acid β-oxidation pathway, which allowed us to assign the gene products to the enzymes involved in this pathway. YwJF might possibly be involved in acyl-CoA dehydrogenation because it is a 4Fe-4S ferredoxin protein. The enzymes of this pathway are long chain fatty acid-CoA ligase (1), acyl-CoA dehydrogenase (22), 3-hydroxyacyl-CoA dehydrogenase/enoyl-CoA hydratase complex (33), acetyl-CoA C-acetyltransferase (4), and 2,4-dienoyl-CoA reductase (55). The boxed and round boxed gene products are under direct and indirect negative control by YsiA, respectively. The doubly encircled long chain acyl-CoAs are inducers interacting with YsiA to prevent the binding of YsiA to YsiA boxes. Dotted boxed Yhfs and Yhfl also exhibited significant similarities to acetyl-CoA C-acetyltransferases and fatty acid-CoA ligases in the BLASTP search (17). The mmgABC genes in parentheses are supposed to encode acetyl-CoA C-acetyltransferase, 3-hydroxyacyl-CoA dehydrogenase/enoyl-CoA hydratase complex, and acyl-CoA dehydrogenase, respectively (13). But these genes are transcribed by c2RNA polymerase (13).
operon, whereas a 1.7-kb one was observed with the yhfL probe, indicating that it is monocistronically transcribed (Fig. 2). As expected, we found palindromic structures, which might act as ρ-independent terminators, just downstream of each of ykuG and yhfL. 5.5- and 7.0-kb transcripts were commonly detected only in the lanes of RNA derived from the ysiA disruptant with the yusL, yusK, or yusJ probe, indicating that the yusL, yusK, and yusJ genes might constitute an operon. Another palindromic sequence was found just downstream of yusJ, so the 5.5-kb transcript covered the yusL, yusK, and yusJ genes. The 7.0-kb transcript, detected with the yusL, yusK, and yusJ probes, appeared to carry the yusM gene besides the yusL, yusK, and yusJ ones (Fig. 2). A 4.2-kb transcript was detected with the ywjF, acdA, and rpoE probes only in lanes of RNA derived from the ysiA disruptant, and a short 0.7-kb transcript was observed in the lanes of RNA derived from both strains FU788 (ysiA::cat) and 168 only with the rpoE probe (Fig. 2). Because an ρ-independent terminator-like sequence was found just downstream of rpoE, the 4.2-kb transcript likely covered the ywjF, acdA, and rpoE genes, whereas the 0.7-kb transcript covered only rpoE.

The lcfA, ysiA, B, etfB, and A genes are clustered in this order. The plasmid pMUTIN integrant in the ysiA gene (strain YSIAd (ysiA::pMUTIN)) (Fig. 4) was constructed to examine ysiA expression. As seen in gene organization of the vicinity of the pMUTIN-disrupted ysiA (Fig. 4), plasmid pMUTIN carrying the 5′-side inside portion of ysiA was integrated into it through single cross-over event; the integration not only disrupts the ysiA gene but also replaces it with lacZ as reporter of ysiA expression (29). Strain YSIAd constitutively synthesized β-Gal (~100 nmol/min/A600) during cell growth in LB medium, indicating that the ysiA gene is autorepressed. Hence, we performed Northern blotting using 2-fold more RNA of strains FU788 (ysiA::cat) and 168 with prolonged 32P exposure of the imaging plates (Fig. 3). So 5.1-, 3.2-, and 0.7-kb transcripts were only detected in the lane of the wild type RNA with the ysiA probe, whereas the 5.1- and 3.2-kb ones were only observed in the lane of the wild type RNA with the ysiB probe, the 0.7-kb
transcript likely coding only ysiA. The 5.1- and 1.8-kb transcripts were only detected in the lane of the wild type RNA with the lcfA probe, the 1.8-kb one appearing to carry only lcfA (Fig. 3). A probable /H9267-independent terminator was found immediately downstream of etfA, so the 3.2-kb transcript probably covers the ysiA, ysiB, etfB, and etfA genes, and the 5.1-kb transcript likely carries the lcfA gene immediate upstream of ysiA besides these four genes. The overall results of Northern blotting indicated that the YsiA regulon comprises five operons (lcfA-ysiA-ysiB-etfB-etfA, ykuF-ykuG, yhfL, yusM-yusL-yusK-yusJ, and ywjF-acdA-rpoE).

To locate the transcription initiation sites for the 3.2-kb ysiA-B-etfBA, 3.5-kb ykuF-G, 1.7-kb yhfL, 5.5-kb yusL-K-J, and 4.2-kb ywjF-acdA-rpoE transcripts, the synthesis of which was highly repressed by YsiA, we performed primer extension analysis using the same RNA samples as those used for Northern analysis (Figs. 2 and 3). As shown in Fig. 5, a specific band of run-off cDNA from each of the primers for the transcripts was strongly repressed by YsiA, so it was only detected with the total RNA from the ysiA disruptant; the RNA fragment corresponding to the 5'-portion of the 3.2-kb ysiA-B-etfBA and 0.7-kb ysiA transcripts was supposed to be abundant in this ysiA disruptant (strain YSIAd). This identification of the transcription initiation bases upstream of the ysiA, ykuF, yhfL, yusL, and ywjF genes allowed us to locate the YsiA-repressive promoters presumably recognized by α4 (Fig. 5). (This identification of the initiation base of the yusL-K-J transcription shifted the initiation codon of YusJ 78 bp downstream compared with the previous assignment (14).) Then we electronically searched for candidate palindromic sequences in the regions upstream of the ysiA, yhfL, ykuF, yusL, and ywjF genes, which might be YsiA boxes. This search revealed palindromic sequences upstream of each gene, the sequences of which are very similar to each other (Fig. 6). It was notable that the putative YsiA boxes for repres-
sion of the \(ysiA-B-\text{etfB-A}, ykuF-G, yusL-K-J, \text{ywjF-acdA-rpoE}\) transcripts. Total RNA samples (45 \(\mu\)g) from strains 168 (wild type) and BFS2426 (ysiA:pMuTN) grown in LB medium to \(A_{600} = 0.5\) were annealed with the respective primers (EysI-A-R, EyhF-L-R, EykuF-R, EysL-R, and EywjF-R; Table 2) for the \(ysiA-B-\text{etfB-A}, yhfL, yusL-K-J, \text{ywjF-acdA-rpoE}\) transcripts, and then primer extension was performed as described in the text. Lanes 1 and 2 contained the run-off DNAs from strains 168 and BFS2426, respectively. Lanes G, A, T, and C contained the products of the respective dideoxy sequencing reactions, performed as described in the text. The arrowheads indicate the run-off DNAs resulting from primer extension analyses. For each analysis, part of the nucleotide sequence of the coding strand corresponding to the ladder is shown together with the transcription initiation base (bold type), and the deduced -10 and -35 regions (underlined). The current identification of the initiation base of the \(yusL-K-J\) transcript allows us to shift the initiation codon (ATG) of the YusL protein to 78 bp downstream of that assigned previously (14).

Identification of YsiA Boxes by in Vitro Analyses—To perform gel retardation analysis to determine whether the putative YsiA boxes function, the YsiA protein was produced in \(E. coli\) and purified by an anion exchange column chromatography to near homogeneity (Fig. 7). The purified YsiA protein exhibited a molecular mass of 40.2 kDa on gel filtration (data not shown), indicating that it is a dimer of two subunits \((M_r = 22.0\) kDa\). On gel retardation analysis involving the purified YsiA protein (Fig. 8A), the \(ysiA\) fragment spanning bases -104 to +143 containing a putative YsiA box for \(ysiA\) (YsiA box\(_{ysiA}\)) became increasingly retarded, forming a band corresponding to a protein-DNA complex, as the amount of YsiA protein in the assay mixture increased. Similarly, the \(ykuF, yhfL, yusL,\) and \(\text{ywjF}\) fragments comprising bases -46 to +123, -65 to +121, -39 to +132, and -98 to +73 with each of putative YsiA boxes for YsiA box\(_{ywjF}\) with was well correlated with their palindrome matching (Fig. 8B).

Identification of Inducers of the YsiA Regulon—\(B. subtilis\)

YsiA is supposed to be a functional homolog of \(FadR\), a global transcriptional regulator of fatty acid metabolism, the inducers of which are long chain acyl-CoA compounds (11). So, we examined whether the interaction of YsiA with a representative YsiA box, YsiA box\(_{ywjF}\), is inhibited by long chain acyl-CoAs (Fig. 10). Branched long chain acyl-CoAs (12-methyltetradecanoyl- and 13-methyltetradecanoyl-CoAs) were chemically synthesized from 12-methyltetradecanoic and 13-methyltetradecanoic acids (15:0), which are the most abundant fatty acids in \(B. subtilis\) (33). As shown in Fig. 10 (A and B), saturated acyl-CoAs with 12–20 carbon atoms but not laurel-CoA, as well as unsaturated oleoyl-CoA (18:1) and palmityloleoyl-CoA (16:1), and branched chain 12-methyltetradecanoyl and 13-methyltetradecanoyl CoAs almost completely inhibited the interaction with YsiA box\(_{ywjF}\) at the concentration of 50 \(\mu\)M, whereas only arachidoyl-CoA, stea-
oyl-CoA, palmitoyl-CoA, oleoyl-CoA, palmitoleoyl-CoA, and 12-metyltetradecanoyl-CoA inhibited it well at the concentration of 5 μM. The equilibrium inhibitor constants (K_i) of the inhibitory acyl-CoAs, calculated from the data obtained in the other gel retardation experiments involving a series of acyl-CoA amounts (data not shown), were 1.0 for aracidoyl-CoA, 0.85 for stearoyl-CoA, 4.3 for palmitoyl-CoA, 5.2 for myristoyl-CoA, 23 for lauroyl-CoA, 0.39 for oleoyl-CoA, 4.0 for palmitoleoyl-CoA, 0.40 for 12-metyltetradecanoyl-CoA, and 5.2 for 13-metyltetradecanoyl-CoA. The results indicated that stearoyl- and oleoyl-CoAs with 18 carbon atoms inhibited this interaction most effectively and that the introduction of one double bond into stearoyl-CoA yielding oleoyl-CoA enhanced this inhibition significantly. Methylation of myristoyl-CoA (tetradecanoyl-CoA) at the 12th carbon also elevated it, 12-metyltetradecanoyl-CoA being one of the best inhibitors. Saturated acyl-CoAs with 3–8 carbon atoms including isovaleryl-CoA did not inhibit this interaction with YsiA box_ywjF. Moreover, neither coenzyme A nor fatty acids inhibited the YsiA interaction with YsiA box_ywjF (Fig. 10). Thus, long chain acyl-CoAs with 14–20 carbon atoms, including oleoyl- and palmitoleoyl-CoAs and 12-metyltetradecanoyl-CoA, are most likely the inducers of the YsiA regulon, interacting with the YsiA protein to prevent the binding to the YsiA boxes.

lacZ Expression under the Control of Each of the YsiA-regulated Promoters in pMUTIN Disruptants—The in vitro experiments suggested that YsiA was a repressor of the YsiA regulon members that interacted with their YsiA boxes to block their transcription and that this interaction was inhibited by long chain acyl-CoAs. Most of the gene products, the synthesis of which is highly repressed by YsiA, are enzymes involved in fatty acid β-oxidation.
To determine whether the disruption of each of them affects their promoter activity, that is, YsiA repression is suppressed by it, we lined up pMutin disruptants of the genes regulated by YsiA (Fig. 4). As seen in gene organization of the vicinity of the pMUTIN-disrupted ysiA, yusL, ywjF, ykuF, and yhfL genes as representatives (Fig. 4), plasmid pMUTIN integration not only disrupts the target gene and replaces it with lacZ as reporter of its expression but also places its downstream genes under the control of the spac promoter which is repressed by E. coli LacI (29). Thus, the addition of IPTG to the medium induces the genes downstream of the disrupted gene.

/H9252-Gal synthesis in a series of pMUTIN disruptants was monitored during the logarithmic and stationary growth phases in the absence and presence of IPTG in the LB medium. Fig. 11A shows the monitoring of /H9252-Gal synthesis in the pMUTIN disruptants of six genes (ysiB, etfB, etfA, yusL, yusK, and yusJ), which are supposed to be directly involved in fatty acid /H9252-oxidation (Fig. 1). As shown in Fig. 11A, /H9252-Gal synthesis in these disruptants started to drastically increase at the beginning of the stationary phase in the absence of IPTG. Even if IPTG was added to the medium, which induces the genes downstream of the disrupted genes, /H9252-Gal synthesis in the disruptants of the yusJ and etfA genes, encoding acyl-CoA dehydrogenase, and the /H9251- and /H9252-subunits of an electron transfer flavoprotein, respectively (Fig. 1), still greatly increased (Fig. 11A), suggesting that these disruptions might cause the accumulation of long chain acyl-CoAs, inducers for the YsiA regulon, in vivo (Fig. 10). We did not detect significant induction in the disruptants as to the yhfL, yusM, yusK, and acdA genes but detected some increase in that of lcfA (Fig. 11B), although transcription of yusM and lcfA was unlikely to be directly regulated by YsiA because of a lack of any YsiA box sequences in their promoter regions (Fig. 6). These
**FIGURE 9.** DNase I footprinting analysis of the interaction of YsiA with the YsiA boxes. The analysis was performed as described in the text. The upper and lower panels are DNase I footprints of the 5′ end-labeled coding and noncoding strands of the DNA probe, respectively. Lanes 1–4 contained 0.04 pmol of the 32P-labeled probe DNA in the reaction mixture (50 μl). Lanes 1–4 contained 0, 44.3, 22.2, and 0 pmol of YsiA (as dimer), respectively. Lanes G, A, T, and C contained the products of the corresponding dideoxy sequencing reactions performed with the same primers as those used for probe preparation. The nucleotide sequences of the protected regions of the coding and noncoding strands of each probe are shown, the sequences of the YsiA boxes being underlined.

**FIGURE 10.** Inhibition of YsiA-specific binding to a YsiA box on the addition of acyl-CoA. The gel retardation analysis was performed as described in the legend to Fig. 8. The reaction mixtures (25 μl) contained 0.02 pmol of the DNA probe containing YsiA box ywfF, which was the same probe as that used in the gel retardation analysis (Fig. 8), 220 nM YsiA protein (as dimer), 5 or 50 μM acyl-CoA or fatty acid (A and B), or 100 μM fatty acid (C), and 4% Me2SO, the concentrations given being final; acyl-CoAs and fatty acids were dissolved in Me2SO. The acyl-CoAs exhibiting a significant inhibitory effect on the formation of the DNA probe-YsiA complex are underlined. The K<sub>i</sub> values of long chain acyl-CoAs are shown in the upper part of A and were obtained in the other gel retardation experiments (data not shown).

*in vivo* results support the above *in vitro* finding that long chain acyl-CoAs are inducers for the YsiA regulon.

**Growth Defect with a Fatty Acid as the Sole Carbon Source Caused by Disruption of Several Members of the YsiA Regulon**—*E. coli fad* genes regulated by FadR are required for cell growth on long chain fatty acids as carbon sources (34). However, no *B. subtilis* mutant unable to grow on long chain fatty acids as the sole carbon source has been reported, as far as we know. Thus, we examined whether disruptants of the YsiA regulon members grew on palmitic acid as the sole
carbon source; we selected this fatty acid arbitrarily, because palmitoyl-CoA was one of the most efficient inducers antagonizing YsiA as to its binding to YsiA boxes, as described above. The cells of each disruptant were spread on plates of a minimal medium (S6) (30) containing 4.35 mM sodium palmitate, well dispersed in this solid medium, as the sole carbon source in the presence of IPTG, followed by incubation at 37 °C. Cell growth between 24 and 48 h was quantitated by measuring $A_{600}$ of the suspensions of the cells grown on plate surfaces. As shown in Fig. 12 (light gray bars), the etfA, B, ykuG, yusL, K, and J disruptants exhibited a clear defect in the utilization of palmitic acid as a carbon source, whereas those of the other genes were able to grow on this fatty acid, as the wild type cells did, a slight growth defect of the ysiB disruptant being observed. IPTG was added to induce the genes downstream of the disrupted gene, so it was strongly suggested that these five genes themselves participated in the utilization of palmitic acid, a representative long chain fatty acid.

To examine whether the genes regulated by YsiA are induced in vivo by the growth on the above minimal medium plates containing palmitic acid as the sole carbon source, we determined the /H9252-Gal activities in the extracts of the cells at 24 h after spreading them on plates when the cells were still viable even if the cells were hardly grown on it. As shown in Fig. 12 (dark gray bars), the promoters directly regulated by YsiA were activated, as observed in the /H9252-Gal activities in the disruptants of ysiB, etfA, etfB, yusL, K, J, ywif, and acdA; /H9252-Gal synthesis was almost negligible in the disruptants of yhfL, ykuF, G, ywjF, and acdA, which were grown in LB medium (Fig. 11B). It is notable that /H9252-Gal synthesis in the disruptants of ysiB, etfA, etfB, yusL, K, J, ywif, and acdA was especially high probably because of inactivation of YsiA by accumulation of long chain acyl-CoAs in the cells. These disruptants except the ysiB disruptant were unable to grow on palmitic acid (Fig. 12); the residual 3-hydroxyacyl-CoA dehydrogenase/enoyl-CoA hydratase activity of YsiB paralogs in the ysiB disruptant might support the growth on palmitic acid, but it might not be enough to prevent the accumulation of long chain acyl-CoAs in the cells.

**FIGURE 11. Derepression of YsiA-repressed genes through their disruption.** The pMUTIN disruptants of the examined genes (strains LCFAd; BFS2426 and BFS2427; ETFAd and ETFBd; YKUFd and BFS1835; YHFLd; BFS1341, BFS1347, BFS1346 and BFS1245; BFS1246; and ACDAd, for lcfA; ysiA, B; etfA, B; ykuF, G; yhfL; yusM, L, K, J; ywif; and acdA, respectively) were grown and incubated for 10 h, and the then β-Gal activities in crude cell extracts were determined, as described in the text. A, the results of monitoring of β-Gal synthesis, obtained for the disruptants of ysiB, etfB, etfA, yusL, yusK, and yusJ, whose products most likely participate in fatty acid β-oxidation, are shown. Open and closed symbols denote $A_{600}$ values and β-Gal activities, and the circles and squares indicate incubation with and without 1 mM IPTG, respectively. B, the β-Gal activities in the extracts of the cells, harvested after 8 h incubation with and without 1 mM IPTG, are indicated by the black and gray bars, respectively. The pMUTIN disruptants of the genes shown under the x axis were used for the monitoring.
Fatty Acid Degradation Genes in B. subtilis

FIGURE 12. Growth of pMUTIN disruptants of the YsiA regulon members on minimal solid medium containing a long chain fatty acid as the sole carbon source and β-Gal synthesis in them. Cells of pMUTIN disruptants of the YsiA regulon members were grown at 30 °C on tryptose blood agar base plus glucose containing erythromycin (0.3 μg/ml) overnight. The cells were suspended in S6 medium (30) containing 50 μg/ml tryptophan. Then a portion of each cell suspension (A600/ml = 0.5) was spread on S6 solid medium containing tryptophan, 4.35 mM sodium palmitate, and 1 mM IPTG and then incubated for 3 days at 37 °C. The cells grown on each of triplicate plates were suspended in 5 ml of S6 medium containing tryptophan for the measurement of A600 every 24 h. The values of ΔA600/plate/24 h on the left y axis are the increases in A600/plate between 24 and 48 h of incubation, which are indicated by light gray bars. The experiments were repeated three times, and the averages of the three values and their standard deviations are shown. The β-Gal activities in the extracts of the cells, suspended at 24 h after spreading them, are indicated by the dark gray bars. The experiments were triplicated, and the three values and their standard deviations are shown.

DISCUSSION

DNA microarray analysis revealed 15 candidate target genes of YsiA, one of the TetR family of bacterial DNA-binding regulatory proteins. A BLASTP similarity search (17) of these products to the nonredundant protein sequence data base revealed that most of the target gene candidates exhibited high similarities to enzymes involved in fatty acid β-oxidation and closely related reactions, suggesting that YsiA might be a central regulator in fatty acid degradation (Fig. 1). Northern blotting indicated that these 15 genes are organized into five operons (lcfA-ysiA-B-etfB-A, ykuF-G, yhfL, yusM-L-K-J, and ywjF-acdA-rpoE) (Figs. 2 and 3). Among many transcripts resulting from these operons, the synthesis of the 5.1-kb lcfA-ysiA-B-etfB-A and 7.0-kb yusM-L-K-J transcripts was under negative control of YsiA without any YsiA box-like sequence in the promoter regions upstream of lcfA and yusM. To explain this indirect transcriptional control through YsiA, we have to assume another transcription regulator whose synthesis and (or) operation is regulated by YsiA, but at present, we have no experimental evidence suggesting what this regulator is.

B. subtilis YsiA is supposed to be a functional homolog of E. coli FadR, a global transcriptional regulator of fatty acid metabolism, the inducers of which are long chain acyl-CoAs (11). As for FadR, long chain acyl-CoAs with 14–20 carbon atoms were considered to be inducers of the YsiA regulon, interacting with the YsiA protein to prevent the binding with the YsiA boxes (Fig. 10). The in vivo experiments involving pMUTIN disruptants of the target genes of YsiA (Fig. 11) revealed that the knock-out of acyl-CoA dehydrogenation through disruption of yusL, etfB, or etfA presumably caused the accumulation of branched and straight long chain acyl-CoAs, which derepressed YsiA-regulated genes. In addition, the disruption of ysiL, yusJ, L, and K directly involved in fatty acid β-oxidation caused the β-Gal induction probably because of the accumulation of long chain acyl-CoAs, when their disruptants were incubated with palmitic acid (Fig. 12); only the ysiB disruptant among them grew on palmitic acid, a representative long chain fatty acid, as the sole carbon source. Fig. 12 also shows that the promoters directly repressed by YsiA are considerably derepressed during cell growth on palmitic acid, as observed in the disruptants of the yhfL, ykuF, ywjF, and acdA promoters, which suggests that moderate inactivation of YsiA by long chain acyl-CoAs likely occurs in the cells growing on palmitic

A or C, and any base, respectively. These sequences can be classified into three groups (ysiA and ykuF; yusL and yhfL; and ywjF) according to the Kd values for their affinity to YsiA and to palindrome matching (Fig. 8B). As seen in comparison between the sequences of YsiA boxysA-B and YsiA boxysL, although such conservation is not seen in that of YsiA boxysF, these base substitutions might be determinants as to the alteration of the Kd values.

A web-based GRASP-DNA search (32) using the sequences of the five YsiA boxes indicated that these sequences were scored as the best five, and there were no more candidate YsiA boxes in the regions of lcfA-ysiA-B-etfB-A, ykuF-G, yusM-L-K-J, ywjF-acdA-rpoE, and yhfL. However, the synthesis of the 5.1-kb lcfA-ysiA-B-etfB-A and 7.0-kb yusM-L-K-J transcripts was under negative control of YsiA without any YsiA box-like sequence in the promoter regions upstream of lcfA and yusM. To explain this indirect transcriptional control through YsiA, we have to assume another transcription regulator whose synthesis and (or) operation is regulated by YsiA, but at present, we have no experimental evidence suggesting what this regulator is.

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B. subtilis BG12330
B. licheniformis BL00330
G. kaustophilus GK6898
B. anthracis BA5192
B. thuringiensis BT9727_4260
B. cereus BC4525
O. iheyensis OB2121
B. halodurans BH3102
B. clausii AR2672

FIGURE 13. High conservation among the YsiA sequences of various Gram-positive low GC bacteria. Alignment of the YsiA sequences of various Gram-positive low GC bacteria is shown. A helix-turn-helix motif comprising 22 amino acids was predicted in the N-terminal region of the YsiA protein (36), which is well conserved among the aligned YsiA proteins.

The lacZ fusion experiments also indicated that YsiA is considered to be able to repress its regulon members during the logarithmic and stationary growth phases in the LB medium, because β-Gal synthesis, except in the pMUTIN integrants as to the YsiA-regulated genes, showed a delay in exiting from the stationary phase (35). However, it is notable that a YkuG, presumably a cell wall protein with a peptidoglycan-binding domain, affected the utilization of palmitic acid. This implies that this protein might be involved in the transport of long chain fatty acids, which is worth investigating. Although the YwjF protein, a 4Fe-4S ferredoxin protein, was considered to be possibly associated with acyl-CoA hydrogenation, we obtained no evidence to support this prediction in the in vivo experiments involving its disruption (Figs. 11 and 12). Furthermore, we have no idea at present how YusM and RpoE participate in fatty acid metabolism, because neither was knock-out nor rpoE expression freed from YsiA-regulation through pMUTIN integration into the genes upstream of rpoE affected aliphatic acid utilization in the presence (Fig. 12) or absence (data not shown) of IPTG.

In E. coli, the FadR protein is a transcription factor that plays a central role in the regulation of fatty acid metabolism, through its functioning as a switch that regulates the machinery required for fatty acid β-oxidation and the expression of a key enzyme in fatty acid biosynthesis (7, 8). Fatty acid metabolism in B. subtilis is likely controlled by FapR (12) (controlling fatty acid biosynthesis and phospholipids) and YsiA (controlling fatty acid degradation). FapR presumably senses the in vivo concentration of malonyl-CoA, an increase of which inactivates this protein (12), whereas YsiA is most likely inactivated by an increase in the in vivo concentration of long chain acyl-CoAs (Figs. 10 and 11), as in the case of E. coli FadR. Interestingly, E. coli FadR is roughly 10 times more sensitive to long chain acyl-CoAs than B. subtilis YsiA (Ref. 11 and Fig. 10), which might reflect the dual regulatory roles of FadR in fatty acid utilization, suggesting that this protein might be involved in the transport of long chain fatty acids, which is worth investigating. Although the YwjF protein, a 4Fe-4S ferredoxin protein, was considered to be possibly associated with acyl-CoA hydrogenation, we obtained no evidence to support this prediction in the in vivo experiments involving its disruption (Figs. 11 and 12). Furthermore, we have no idea at present how YusM and RpoE participate in fatty acid metabolism, because neither was knock-out nor rpoE expression freed from YsiA-regulation through pMUTIN integration into the genes upstream of rpoE affected aliphatic acid utilization in the presence (Fig. 12) or absence (data not shown) of IPTG. Nevertheless, it is notable that a rpoE mutant strain has an altered morphology and shows a delay in exiting from the stationary phase (35).

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acid synthesis and degradation, possibly requiring finer tuning of this coordinate regulation.

The BLASTP similarity search (17) also revealed that YsiA is highly conserved in many Gram-positive organisms including Bacillus, Clostridium, Streptomyces, and other related genera; alignment of the YsiA sequences of Bacillus and closely related genera is shown in Fig. 13. Moreover, its orthologs were unexpectedly found in far-diverse genera, such as Metanosarcina (Archaea), and Bordetella, Burkholderia, and Chromobacteria (betaproteobacteria). It is noteworthy that FapR, a global transcription factor for membrane lipid biosynthesis, is conserved in the Bacillus, Listeria, and Staphylococcus genera and also in Clostridium and other related genera (12), but YsiA is not conserved in the Listeria and Staphylococcus genera. This diverse but unique distribution of YsiA orthologs implies that horizontal gene transfer(s) might have occurred during its molecular evolution.

In the N-terminal region of B. subtilis YsiA (Fig. 13), a helix-turn-helix motif covering amino acids 27–48 was predicted with NPS@ (network protein sequence analysis) (36). In the framework of this coordinate regulation, the tridium...
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