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Dual Regulation of the *Bacillus subtilis* Regulon Comprising the *lmrAB* and *yxaGH* Operons and *yxaF* Gene by Two Transcriptional Repressors, LmrA and YxaF, in Response to Flavonoids[▽]

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Bacillus subtilis **LmrA is known to be a repressor that regulates the *lmrAB* and *yxaGH* operons; *lmrB* and *yxaG* encode a multidrug resistance pump and quercetin 2,3-dioxygenase, respectively. DNase I footprinting analysis revealed that LmrA and YxaF, which are paralogous to each other, bind specifically to almost the same *cis* sequences, LmrA/YxaF boxes, located in the promoter regions of the *lmrAB* operon, the *yxaF* gene, and the *yxaGH* operon for their repression and containing a consensus sequence of AWTATAtagaNYGgTCTA, where W, Y, and N stand for A or T, C or T, and any base, respectively (three-out-of-four match [in lowercase type]). Gel retardation analysis indicated that out of the eight flavonoids tested, quercetin, fisetin, and catechin are most inhibitory for LmrA to DNA binding, whereas quercetin, fisetin, tamarixetin, and galangin are most inhibitory for YxaF. Also, YxaF bound most tightly to the tandem LmrA/YxaF boxes in the *yxaGH* promoter region. The *lacZ* fusion experiments essentially supported the above-mentioned in vitro results, except that galangin did not activate the *lmrAB* and *yxaGH* promoters, probably due to its poor incorporation into cells. Thus, the LmrA/YxaF regulon presumably comprising the *lmrAB* operon, the *yxaF* gene, and the *yxaGH* operon is induced in response to certain flavonoids. The in vivo experiments to examine the regulation of the synthesis of the reporter β-galactosidase and quercetin 2,3-dioxgenase as well as that of multidrug resistance suggested that LmrA represses the *lmrAB* and *yxaGH* operons but that YxaF represses *yxaGH* more preferentially.**

Bacteria in soil are exposed to various deleterious compounds released by microbes, plants, and animals. To prevent the invasion of such compounds into their cells, they employ an efflux system involving multidrug resistance (MDR) pumps that shows broad substrate specificity. In a gram-positive soil bacterium, *Bacillus subtilis*, seven MDR pumps have been identified so far and classified into two groups. Bmr, Blt, Bmr3, and LmrB belong to the major facilitator superfamily, while BceAB, BmrA, and VmlR are ABC transporters. One of the members of the *B. subtilis* major facilitator superfamily, LmrB, is involved in resistance to several drugs such as lincomycin and puromycin (14). The *lmrB* gene is the second gene of the *lmrAB* operon, and the first gene, *lmrA*, encodes a transcriptional repressor for *lmrAB*, which belongs to the TetR family (17).

Our previous study showed that LmrA interacts with a *cis* sequence in the *lmrAB* promoter region. With the combination of DNA microarray analysis and genome-wide computational sequence analysis, another LmrA target, *yxaGH*, was identified, which was found in the putative *yxaGH* promoter region two tandem LmrA-binding *cis* sequences, each of which is similar to that in the *lmrAB* promoter region (30). However, the DNA binding of LmrA was not inhibited by lincomycin, with the inducer interacting with LmrA remaining to be identified (30). Besides these findings, it was reported that *yxaG* encodes quercetin 2,3-dioxygenase, which is involved in flavonol degradation (3), whereas *yxaH* encodes a membrane protein of unknown function.

Flavonoids including quercetin, which are considerably abundant in soil, are secreted from the roots of higher plants and released through the degradation of plant cells. Some flavonoids possess antibacterial activity; quercetin inhibits bacterial DNA gyrase, which induces DNA cleavage (16, 24). To avoid such harmful effects, some bacteria are equipped with a degradation system for flavonoids for detoxification (18). Hence, the gene product of *yxaG*, encoding quercetin 2,3-dioxygenase, appears to play a major role in flavonoid degradation in *B. subtilis*.

LmrA is paralogous to YxaF, the gene of which is located immediately upstream of the *yxaGH* operon and oriented in the same direction as *yxaGH* (Fig. 1). On the assumption that LmrA and YxaF might recognize and bind to almost the same *cis* sequences located in the promoter regions of the target genes, we performed in vitro and in vivo experiments to characterize the operon organization of the regulon regulated by LmrA and YxaF, the LmrA/YxaF regulon. We also carried out experiments to identify the *cis* sequences to which LmrA and YxaF bind, i.e., LmrA/YxaF boxes, and to determine if some flavonoids such as quercetin might be inducers for the LmrA/YxaF regulon.

We report in this communication that the LmrA/YxaF regu-

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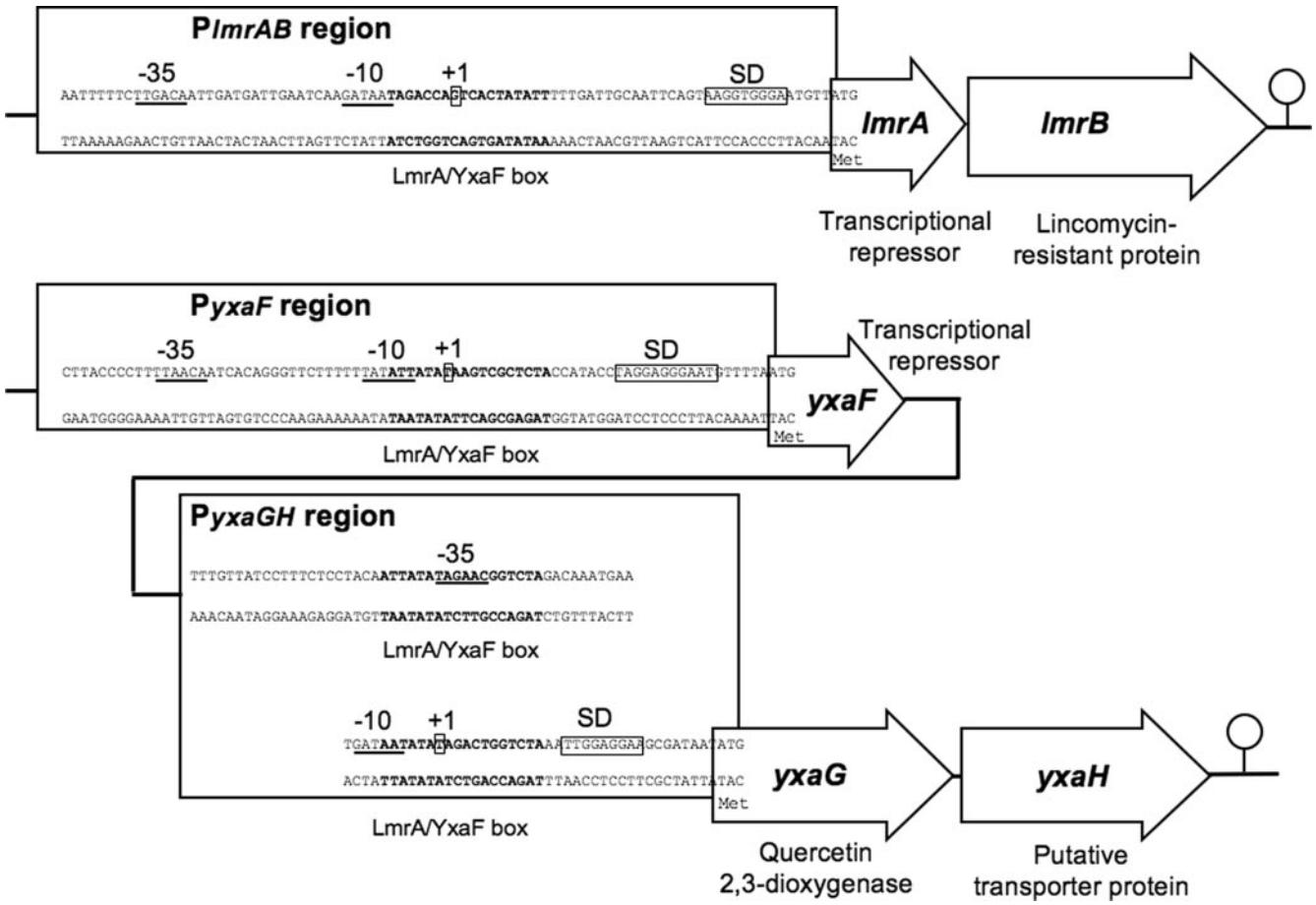


FIG. 1. Organization of the LmrA/YxaF regulon comprising the *lmrAB* operon, the *yxaF* gene, and the *yxaGH* operon and their promoter regions. The five genes are depicted as thick open arrows, and two hairpin structures likely functioning as ρ-independent transcription terminators are illustrated. The −35 and −10 regions for each operon and gene are underlined, and their transcription start sites (+1) and Shine-Dalgarno (SD) sequences are boxed. The LmrA/YxaF boxes comprising 18 bp are shown in boldface type; the box sequence in the *lmrAB* promoter region is reversed.

lon presumably comprises the *lmrAB* operon, the *yxaF* gene, and the *yxaGH* operon. The LmrA/YxaF boxes are located in their promoter regions, the binding of LmrA and YxaF to which was inhibited effectively but distinctly by such flavonoids as quercetin, fisetin, tamarixetin, galangin, and catechin. This is the first demonstration of a regulation system of the *Bacillus* genus that responds to certain flavonoids that are common in soil. Moreover, it is notable that YxaF preferentially binds to the tandem LmrA/YxaF boxes in the *yxaGH* promoter region. We discuss the physiological meaning of the dual regulation of the LmrA/YxaF regulon through LmrA and YxaF and the relation of MDR and flavonoid degradation.

MATERIALS AND METHODS

***B. subtilis* strains and their construction and cultivation.** The *B. subtilis* strains used in this study are listed in Table 1. *B. subtilis* strain 168 was used as the wild-type strain. Strain PLR2 (14) was provided by M. Murata (Kyorin University, Japan). Strains LMRAd, LMRBd, YXAFd, YXAGd, and YXAHd were constructed by the integration of plasmid pMUTIN2 (25) into the *lmrA*, *lmrB*, *yxaF*, *yxaG*, and *yxaH* genes in strain 168, respectively (10) (refer to the Japan Functional Analysis Network for *B. subtilis* [JAFAN] website [http://bacillus.genome.jp]). Strain FU778 was constructed in the framework of JAFAN by the transformation of strain 168 with a DNA fragment generated through long-

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

Strain	Genotype	Reference or source
168	*trpC2*	Laboratory stock (wild type)
PLR2	*lmrA*(*Q52P stop to S*) *trpC2*	14
LMRAd	*lmrA*::pMUTIN2 *trpC2*	10
LMRBd	*lmrB*::pMUTIN2 *trpC2*	10
YXAFd	*yxaF*::pMUTIN2 *trpC2*	10
YXAGd	*yxaG*::pMUTIN2 *trpC2*	10
YXAHd	*yxaH*::pMUTIN2 *trpC2*	10
FU778	Δ*lmrA*::*cat trpC2*	JAFAN
FU875	Δ*yxaF*::*cat trpC2*	This study
FU876	Δ*lmrA*::*tet trpC2*	This study
FU877	Δ*yxaF*::*cat lmrA*::pMUTIN2 *trpC2*	This study
FU878	Δ*yxaF*::*cat lmrB*::pMUTIN2 *trpC2*	This study
FU879	Δ*yxaF*::*cat yxaG*::pMUTIN2 *trpC2*	This study
FU880	Δ*lmrA*::*tet yxaG*::pMUTIN2 *trpC2*	This study
FU881	Δ*lmrA*::*tet* Δ*yxaF*::*cat yxaG*::pMUTIN2 *trpC2*	This study
FU899	Δ*yxaF*::*cat lmrA*(*Q52P stop to S*) *trpC2*	This study
FU967	Δ*lmrA*::*tet* Δ*yxaF*::*cat yxaH*::pMUTIN2 *trpC2*	This study

TABLE 2. Oligonucleotide primers used in this study

Primer	Sequence (5′–3′)[a]
LmrAN	GGTGGGAATCATATGAGTTATG
LmrAB[b]	CGCGGATCCATGCTTTAGCTGTTGTTTCC
YxaFE	CCGGAATTCAGTCGCTCTACCATACCTAG
YxaFN	GGGAATGTTCATATGACTAGCA
YxaFB	CGCGGATCCTCCTCCAATTTAGACCAGTC
MK1[b]	CAGTCAAAAGATCAGTCAGC
MK2[b]	TGCCGTAATACCCTTGCAGC
PyxaG1[b]	GGTGAGGAAAAAAGGGTAGC
PyxaG2[b]	CTCCGGAGCAAATAAGGCAT
PyxaF1	CTGTTGTTCAAGCTGCTCTG
PyxaF2	TTCACGTGAATCTCCTCTGC
yxaFupF	CTGCCGGTGTAGTGTTTGACAGC
yxaFupR_catdown	GAGATAATGCCGACTGTACTGTGAATCTCCTCTGCTAGTCAT
yxaFdownF_catup	CTAATGGGTGCTTTAGTTGAAGAACCAGTGCTGGTGAGGAAAAAAGGG
yxaFdownR	CAATTGTCGCTACAGCGGCTGC
catF	TCTTCAACTAAAGCACCCATTAG
catR	AGTACAGTCGGCATTATCTC
yxaFupF2	CGCTCTTGTTCCGTAAACAGGTCC
yxaFdownR2	AAATCGCTCGTTTGAGACTTCTTCG

[a] Each restriction enzyme site is underlined.
[b] Primers are the same as those used in a previous study (30).

flanking homology PCR (26) involving the chloramphenicol acetyltransferase gene (*cat*) (8) as a selective marker, which was provided by K. Kobayashi (Nara Institute of Science and Technology, Japan). The *cat* gene is oriented in the same direction as the original *lmrA*.

B. subtilis strain FU875, carrying a *yxaF* deletion, was constructed as follows. Long-flanking homology PCR (26) was performed to create a DNA fragment in which the *cat* gene (8), in the reverse orientation, was sandwiched by the upstream and downstream regions including the short 5′ and 3′ ends of the *yxaF* gene, respectively. The two regions upstream and downstream of *yxaF* were amplified by PCR with the genomic DNA of strain 168 as a template and two primer pairs (yxaFupF/yxaFupR_catdown and yxaFdownF_catup/yxaFdownR, respectively) (Table 2). The *cat* cassette was amplified by PCR with a primer pair (catF/catR) (Table 2) and DNA of plasmid pCBB31 bearing the *cat* gene (K. Kobayashi, unpublished data) as a template. The above-mentioned three PCR products were mixed into the reaction mixture containing ExTaq DNA polymerase (Takara-Bio, Japan) and deoxynucleoside triphosphates without any primer oligonucleotide, and denaturation, annealing, and extension reactions were the carried out to combine the three fragments. Nested PCR with the resultant fragment as a template and a primer pair (yxaFupF2/yxaFdownR2) (Table 2) was performed to amplify the combined DNA fragment, which was then used to transform strain 168 to chloramphenicol resistance (5 μg/ml) to obtain strain FU875 (Table 1). The correct replacement of the *yxaF* gene with *cat* was confirmed by means of PCR and DNA sequencing.

Strain FU778 was transformed with plasmid pCm::Tc (22) to change chloramphenicol resistance to tetracycline resistance (10 μg/ml) to yield strain FU876 (Table 1). Strains LMRAd, LMRBd, YXAGd, and PLR2 were transformed with the genomic DNA of strain FU875 to obtain chloramphenicol resistance to result in strains FU877, FU878, FU879, and FU899 (Table 1), respectively. Strains YXAGd and FU879 were transformed with the genomic DNA of FU876 to obtain tetracycline resistance to yield strains FU880 and FU881 (Table 1), respectively. Strain YXAHd was transformed with the genomic DNAs of strains FU875 and FU876 to obtain tetracycline and chloramphenicol resistances to yield strain FU967.

B. subtilis cells were pregrown on plates with tryptose blood agar base (Difco) supplemented with 0.18% glucose (TBABG) containing chloramphenicol (5 μg/ml), erythromycin (0.3 μg/ml), tetracycline (10 μg/ml), and/or lincomycin (25 μg/ml), according to drug resistance, at 30°C overnight. The cells were inoculated into Luria-Bertani (LB) medium (19) and then incubated at 37°C with shaking.

Overexpression of the *lmrA* and *yxaF* genes in *Escherichia coli* and LmrA and YxaF preparation. *E. coli* strain JM109 bearing plasmid pLMRA, a pUC18-based plasmid carrying the *lmrA* gene, was used for *lmrA* expression, the construction of which was described previously (30). To construct plasmid pET-LMRA, a pET-22b(+)-based plasmid carrying the *lmrA* gene, a region containing *lmrA* was amplified by PCR with genomic DNA of *B. subtilis* strain 168 as a template and primer pair LmrAN/LmrAB (Table 2), digested with NdeI and BamHI, and then ligated with vector pET-22b(+), which had been treated with the same restriction enzymes. The ligated DNA was used for the transformation of *E. coli* strain JM109 to ampicillin resistance (50 μg/ml) to produce an expression plasmid, pET-LMRA.

To clone the *B. subtilis yxaF* gene into plasmids pUC18 and pET-22b(+), the two *yxaF* fragments were amplified by PCR with genomic DNA of strain 168 as a template and the primer pairs YxaFE/YxaFB and YxaFN/YxaFB, respectively (Table 2). The resultant PCR products were digested with EcoRI/BamHI and NdeI/BamHI and then ligated with the arms of plasmids pUC18 and pET-22b(+) digested with the same restriction enzymes, respectively. Also, the ligated mixtures were used for the transformation of strain JM109 to ampicillin resistance to yield two expression plasmids, pYXAF and pET-YXAF, respectively. The correct cloning of the *lmrA* and *yxaF* genes in all the resultant plasmids was confirmed by DNA sequencing.

E. coli strain JM109 bearing plasmid pLMRA or pYXAF and strain BL21(DE3) transformed with plasmid pET-LMRA or pET-YXAF were grown in 50 ml of LB medium supplemented with ampicillin (50 μg/ml) at 37°C to an optical density at 600 nm (OD_{600}) of 0.6. After isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, the culture was further cultivated for 3 h. The cells were harvested and then disrupted by sonication in 20 mM Tris-Cl buffer (pH 8.0) containing 10% (vol/vol) glycerol, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol. After centrifugation (17,000 × *g* for 30 min), the supernatants were recovered as crude extracts for the in vitro binding analyses of LmrA and YxaF. The protein concentrations of the crude extracts were measured by a method described previously Bradford (4) using bovine serum albumin as a standard.

Primer extension analysis. Primer extension analysis to determine the transcription start sites of the *yxaF* gene and the *yxaGH* operon was performed essentially as described previously (20, 29). Quercetin dissolved in dimethyl sulfoxide (DMSO) was added to the culture in the logarithmic growth phase (OD_{600} of 0.2) to give 200 μg/ml, which was followed by a further 1 h of cultivation; the same volume of DMSO as that of the quercetin solution was also added to another control culture. Total RNA was extracted from the harvested cells and then purified as described previously (29). For primer extension of the *yxaF* gene and *yxaGH* transcripts, total RNA (45 μg) was annealed to 1 pmol each of primers PyxaF2 and PyxaG2 (Table 2), which had been 5′-end labeled with a MEGALABEL kit (Takara-Bio) and [γ-^{32}P]ATP (GE Healthcare). The primer extension reaction was conducted with ThermoScript reverse transcriptase (Invitrogen). Templates for the dideoxy sequencing reactions for ladder preparation, starting from the same 5′-end-labeled primers as those for *yxaF* and *yxaGH*, were generated by PCR with genomic DNA of strain 168 as a template and primer pairs PyxaF1/PyxaF2 and PyxaG1/PyxaG2, respectively (Table 2). Autoradiograms were obtained and quantified using a Typhoon 9400 variable image analyzer (GE Healthcare).

DNase I footprinting analysis. DNase I footprinting experiments were performed essentially as described previously (6, 30). The *lmrAB*, *yxaGH*, and *yxaF* probes for the footprinting analyses were prepared by PCR with genomic DNA of strain 168 as a template and primer pairs MK1/MK2, PyxaG1/PyxaG2, and PyxaF1/PyxaF2 (Table 2), respectively. Prior to the PCR amplification, only the 5′ terminus of one of the primer pairs was labeled with [γ-^{32}P]ATP using the MEGALABEL kit.

Gel retardation analysis. The gel retardation analysis was performed essentially as described previously (27, 30). The LmrA and YxaF extracts prepared from BL21(DE3) cells bearing pET-LMRA and pET-YXAF, respectively, were dialyzed against the same buffer as that used for the extract preparation to eliminate contaminating small molecules from the cells, which might affect the DNA binding of the protein. The amounts of LmrA and YxaF compared to total protein in the dialyzed extracts were estimated by measuring the intensities of the bands on the polyacrylamide gel stained with Coomassie brilliant blue using NIH Image (http://rsb.info.nih.gov/nih-image/). The same *lmrAB*, *yxaGH*, and *yxaF* probes as those used for DNase I footprinting were labeled by PCR in the presence of [α-^{32}P]dCTP (MP Biomedicals) with the same primer pairs as those used for the probe amplification.

DNA microarray analysis. DNA microarray analysis was performed as described previously (11, 15, 29). RNA samples were prepared from cells of strains

168 and FU877 that had been grown in LB medium (19) and harvested in the middle of logarithmic growth at an OD_{600} of 0.5.

***lacZ* fusion experiments to monitor *lmrAB* and *yxaGH*.** Cells pregrown on TBABG plates containing an antibiotic(s) were inoculated into 50 ml of LB medium at an initial OD_{600} of 0.05 and then incubated at 37°C with shaking. When the OD_{600} reached 0.2, each of the flavonoids dissolved in DMSO was added to the medium to give a final concentration of 200 μg/ml, corresponding to 0.6, 0.7, 0.6, 0.7, 0.6, 0.7, 0.8, and 0.7 mM for quercetin, fisetin, tamrixetin, galangin, (+)-catechin, genistein, daidzein, and coumestrol, respectively. As a control, 200 μl of DMSO was added instead of the flavonoid solution. One-milliliter aliquots of the culture were then withdrawn at intervals of 1 h, and the β-galactosidase (β-Gal) activity in crude cell extracts was spectrophotometrically measured by using *o*-nitrophenyl-β-D-galactopyranoside (Wako Pure Chemicals Industries, Japan) as a substrate, according to a procedure described previously (1). To reduce the chromatic disturbance of the β-Gal assay by the flavonoid that adhered to the cells, the latter was washed with 100 mM phosphate buffer (pH 7.5) before lysozyme treatment.

Flavonoid screening. Cells of strain YXAGd were pregrown in LB medium containing 0.3 μg/ml erythromycin overnight at 25°C. The cells were spread well onto TBABG plates containing 50 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside, onto which paper disks soaked in 10 mM each of the various flavonoids were placed, and the plates were then incubated at 37°C. The blue coloration around the disk(s), resulting from the synthesis of β-Gal regulated by LmrA and YxaF exposed to the flavonoids, was monitored.

Assay of lincomycin resistance. The lincomycin resistance of *B. subtilis* cells was assayed by a method described previously (14). Cells pregrown on TBABG plates were inoculated to an OD_{590} of 0.05 in 200 μl of LB medium containing serially diluted lincomycin solutions in the wells of a 96-well titer plate. The growth of cells incubated with shaking was monitored by measuring the OD_{590} 4 h after inoculation, when the cells had reached the late logarithmic phase in medium containing no lincomycin. The concentration of lincomycin that resulted in 50% growth inhibition (IC_{50}) was determined. Experiments were also conducted in the presence of quercetin (0.3 mM) to examine its effect on lincomycin resistance.

Assay of quercetin 2,3-dioxygenase. *B. subtilis* cells pregrown on TBABG plates were inoculated into 50 ml of LB medium to an OD_{600} of 0.05 and then cultivated at 37°C with shaking. When the OD_{600} reached 0.8, the cells were harvested and washed with 50 mM Tris-Cl buffer (pH 7.5) containing 100 mM NaCl, 20 mM NH_4Cl, and 1% (vol/vol) glycerol. The cells were then suspended in the same buffer containing 50 μg/ml lysozyme and 5 μg/ml DNase I and incubated at 37°C for 20 min. After centrifugation (17,000 × *g* for 30 min), the supernatant was recovered as a crude extract. The protein concentrations of the crude extracts prepared were measured as described above.

Quercetin 2,3-dioxygenase activity was determined by measuring the decrease of the absorbance at the characteristic spectroscopic peak of quercetin (ε_{367} = 22,700 $M^{-1}cm^{-1}$ at pH 7.5) due to quercetin decomposition, as reported previously (2). For each assay, 870 μl of 50 mM Tris-Cl buffer (pH 7.5) was added to a quartz cell. Quercetin dissolved in DMSO (30 μl) was then mixed to give 45 μM, followed by preincubation at 25°C for 5 min. The enzymatic reaction was started by adding 100 μl of a crude extract (10 to 100 μg total protein) to the mixture, and the absorbance at 367 nm was monitored at 25°C.

Flavonoids. Quercetin, fisetin, (+)-catechin, genistein, daidzein, 3-hydroxyflavone, myricetin, morin, and kaempferol were products from Sigma. Tamarixetin and galangin were purchased from Nacalai Tesque (Japan), coumestrol was obtained from Fluka, and robinetin was obtained from Extrasynthese SA.

RESULTS

Cloning and overexpression of the *lmrA* and *yxaF* genes. Comparison of the amino acid sequences of the LmrA and YxaF proteins revealed 56% identity, with their N-terminal regions including the helix-turn-helix (HTH) motif responsible for DNA binding (5, 17, 21) being especially conserved, implying that these two repressor proteins might recognize and bind to similar *cis* sequences (Fig. 2). In order to prepare LmrA and YxaF extracts for the in vitro experiments, the corresponding genes were cloned into vector pUC18 or pET-22b(+), and overproduction of LmrA and YxaF was achieved by IPTG induction, as described above. During the cloning of *yxaF*, we found two presumable misreadings in the *yxaF* sequence that were previously reported (13), which resulted in the substitution of serine 19 with phenylalanine in the amino acid sequence of YxaF (Fig. 2).

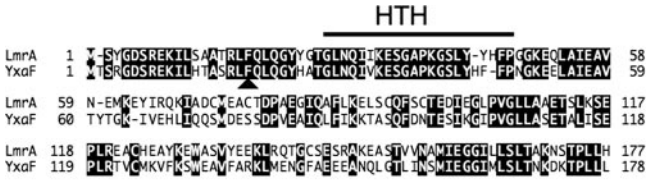


FIG. 2. Alignment of the amino acid sequences of LmrA and YxaF. The conserved residues are boxed in dark gray. During the cloning of *yxaF* in *E. coli* plasmids, the bases at the 56th and 564th positions from the translation start site were found to be T and A (not C and G), respectively, resulting in the substitution of serine 19 with phenylalanine (indicated by an arrowhead). The HTH motifs of the two proteins, which were predicted using NPS@ (network protein search analysis) (5) and deduced from the crystal structure of YxaF (21), are indicated by a horizontal bar.

Determination of the binding sites for LmrA and YxaF in the *lmrAB*, *yxaGH*, and *yxaF* promoter regions. First, we tried to determine the transcription start sites of the *yxaF* gene and the *yxaGH* operon by primer extension analysis; the transcription units of *lmrAB* and *yxaGH* as well as the transcription start site of the *lmrAB* operon were determined previously (12, 28). RNA samples were prepared from cells of strain 168 grown in LB medium with and without quercetin (200 μg/ml), with the expectation that quercetin might induce the *yxaGH* operon because *yxaG* encodes quercetin 2,3-dioxygenase (3). As shown in Fig. 3, the respective specific bands of runoff cDNAs resulting from *yxaF* and *yxaGH* mRNAs were detected only with the RNAs from the cells grown with quercetin. The results allowed us to identify the transcription initiation sites of *yxaF* and *yxaGH* and also indicated that the transcription of *yxaF* and *yxaGH* was actually induced by quercetin. Therefore, we predicted that the −35 and −10 regions of the *yxaF* and *yxaGH* promoters are TTAACA and TATATT, and TAGAAC and GATAAT, with 17-bp spacers, respectively (Fig. 1), which are likely recognized by σ^A RNA polymerase (7).

DNase I footprinting analysis was performed using LmrA and YxaF extracts prepared from cells of strain JM109 carrying plasmids pLMRA and pYXAF, respectively, which had been exposed to IPTG. When the LmrA extract was mixed with the *lmrAB* probe (bases −120 to +118) and the *yxaGH* probe (bases −81 to +90) (+1; each of the transcription start bases of *lmrAB* and *yxaGH*), LmrA protected one region in the *lmrAB* promoter and two regions in the *yxaGH* promoter against DNase I (bases −12 to +11 of the coding strand and bases −14 to +12 of the noncoding strand of *lmrAB* and bases −40 to −11 and −6 to +19 of the coding strand and bases −42 to −14 and −10 to +17 of the noncoding strand of *yxaGH*, respectively) (Fig. 4), which is consistent with results reported previously (30). We then carried out DNase I footprinting experiments using the YxaF extract. As a result, YxaF was found to specifically protect almost the same regions as LmrA did (bases −13 to +10 of the coding strand and bases −12 to +12 of the noncoding strand for *lmrAB* and bases −39 to −11

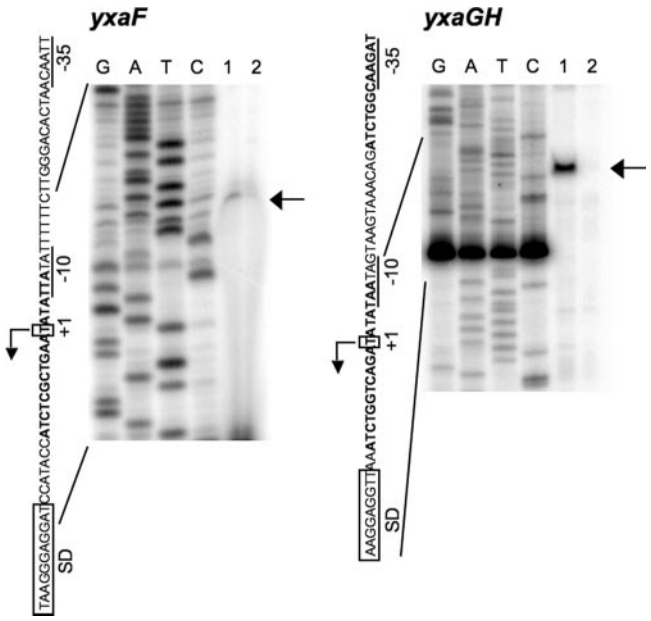


FIG. 3. Determination of the transcription start sites of the *yxaF* gene (left) and the *yxaGH* operon (right) by primer extension analysis. Total RNAs of strain 168 cells grown in the presence (lane 1) or absence (lane 2) of quercetin were prepared and used for the reverse transcription reactions to generate the runoff cDNAs, as described in Materials and Methods. Lanes G, A, T, and C contain the products of the dideoxy sequencing reactions with the same primers as those used for the respective reverse transcriptions. The runoff cDNAs are indicated by arrows. The partial nucleotide sequences of the coding strands corresponding to the ladders are shown, where the −35 and −10 regions are underlined and the transcription start sites (+1) and the Shine-Dalgarno (SD) sequences are boxed.

and −6 to +20 of the coding strand and bases −42 to −14 and −10 to +17 of the noncoding region for *yxaGH*) (Fig. 4).

We also conducted DNase I footprinting to determine if LmrA and YxaF bind to the *yxaF* promoter region. When the LmrA and YxaF extracts prepared from cells of strain BL21(DE3) bearing plasmids pET-LMRA and pET-YXAF were mixed with the DNA probes corresponding to bases −142 to +63 (+1 is the transcription start base of *yxaF*), the two proteins protected the same regions (bases −6 to +19 of the coding strand and bases −9 to +14 of the noncoding one) (Fig. 4). These results revealed that not only the *lmrAB* and *yxaGH* operons but also the *yxaF* gene is a member of the LmrA/YxaF regulon. Also, the four LmrA/YxaF boxes, comprising 18 bp and containing a consensus sequence of AWTATAtagaNYGg TCTA, where W, Y, and N stand for A or T, C or T, and any base, respectively (three-out-of-four match [in lowercase type]), were identified in the promoter regions of *lmrAB*, *yxaGH*, and *yxaF* (Fig. 1). (This consensus sequence is complementary to the previously proposed sequence [30]; the newly proposed sequence is in the same orientation as three out of the four LmrA/YxaF box sequences currently identified.)

Quercetin was found to trigger *yxaF* and *yxaGH* transcription (Fig. 3), implying that it might be an inducer for the LmrA/YxaF regulon. When the DNase I footprinting experiments were carried out in the presence of 1.5 mM quercetin, the specifically protected regions of *lmrAB*, *yxaGH*, and *yxaF* disappeared (Fig. 4, lanes 6). These results clearly indicated that quercetin prevents the binding of LmrA and YxaF to the *lmrAB*, *yxaGH*, and *yxaF* LmrA/YxaF boxes, implying that quercetin might function as an inducer for the LmrA/YxaF regulon.

Quantitative analysis of quercetin inhibition of the LmrA and YxaF binding to the LmrA/YxaF boxes. To analyze quercetin inhibition of LmrA and YxaF binding to the LmrA/YxaF boxes quantitatively, we performed gel retardation analysis involving the LmrA or YxaF protein and each of the same *lmrAB* and *yxaGH* probes as those used for DNase I footprinting (Fig. 4). We attempted to purify the LmrA and YxaF proteins to near homogeneity but failed to obtain pure active LmrA due to its instability. Instead, we used the LmrA and YxaF preparations of 38 and 35% purity, respectively, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) (data not shown), for this gel retardation analysis, which had been dialyzed to remove small molecules derived from the cell extracts.

As shown in Fig. 5A, both LmrA and YxaF bound to each of the *lmrAB* and *yxaGH* probes containing the *lmrAB* and *yxaGH* promoter regions, which produced the retarded bands detected by PAGE. The binding of LmrA and YxaF to the *lmrAB* probe was weaker than that to *yxaGH*, and LmrA binding to the two probes was weaker than that of YxaF. (The binding and association constants for LmrA and YxaF binding to the *lmrAB* and *yxaGH* probes were very roughly estimated to be 15 and 5 nM, and 10 and 1 nM, respectively, as dimers [Fig. 5A]; LmrA and YxaF presumably each exist as homodimers, as deduced from the crystal structure of YxaF [21].) When YxaF was mixed with the *yxaGH* probe, two steps of retardation were observed, implying that each YxaF dimer might bind stepwise to the two LmrA/YxaF boxes in the *yxaGH* promoter. However, the binding of LmrA to the *yxaGH* probe did not exhibit such a stepwise retardation pattern clearly.

We attempted to determine the equilibrium inhibitor constants (K_i) of quercetin for LmrA and YxaF binding to each of the *lmrAB* and *yxaGH* probes with 138 nM LmrA and 125 nM YxaF (Fig. 5B), which are sufficient to cause the complete retardation of the probes (Fig. 5A). When gel retardation was analyzed with different concentrations of quercetin (4.9 μM to 10 mM) (Fig. 5B), it was found that that the binding of the two repressors to the *yxaGH* probe is more tolerant to quercetin than that to *lmrAB* and that YxaF is likely more sensitive to quercetin than LmrA is (Fig. 5B). Moreover, the stepwise retardation pattern was not clearly observed for the quercetin inhibition of YxaF binding to the *yxaGH* probe (Fig. 5B), although it was observed in the absence of quercetin (Fig. 5A). We cannot explain this difference properly.

Relief from repression of the *lmrAB and yxaGH* promoters in vivo by LmrA and YxaF upon quercetin addition. Since quercetin was found to inhibit the binding of LmrA and YxaF to the LmrA/YxaF boxes in vitro, we examined, through *lacZ* fusion experiments, if it actually functions as an inducer for the LmrA/YxaF regulon, i.e., if it releases the repression of the *lmrAB* and *yxaGH* promoters through the LmrA and YxaF repressors. Plasmid pMUTIN2 integration not only disrupts the target gene and replaces it with *lacZ* as a reporter of its expression, it also places its downstream genes under the con-

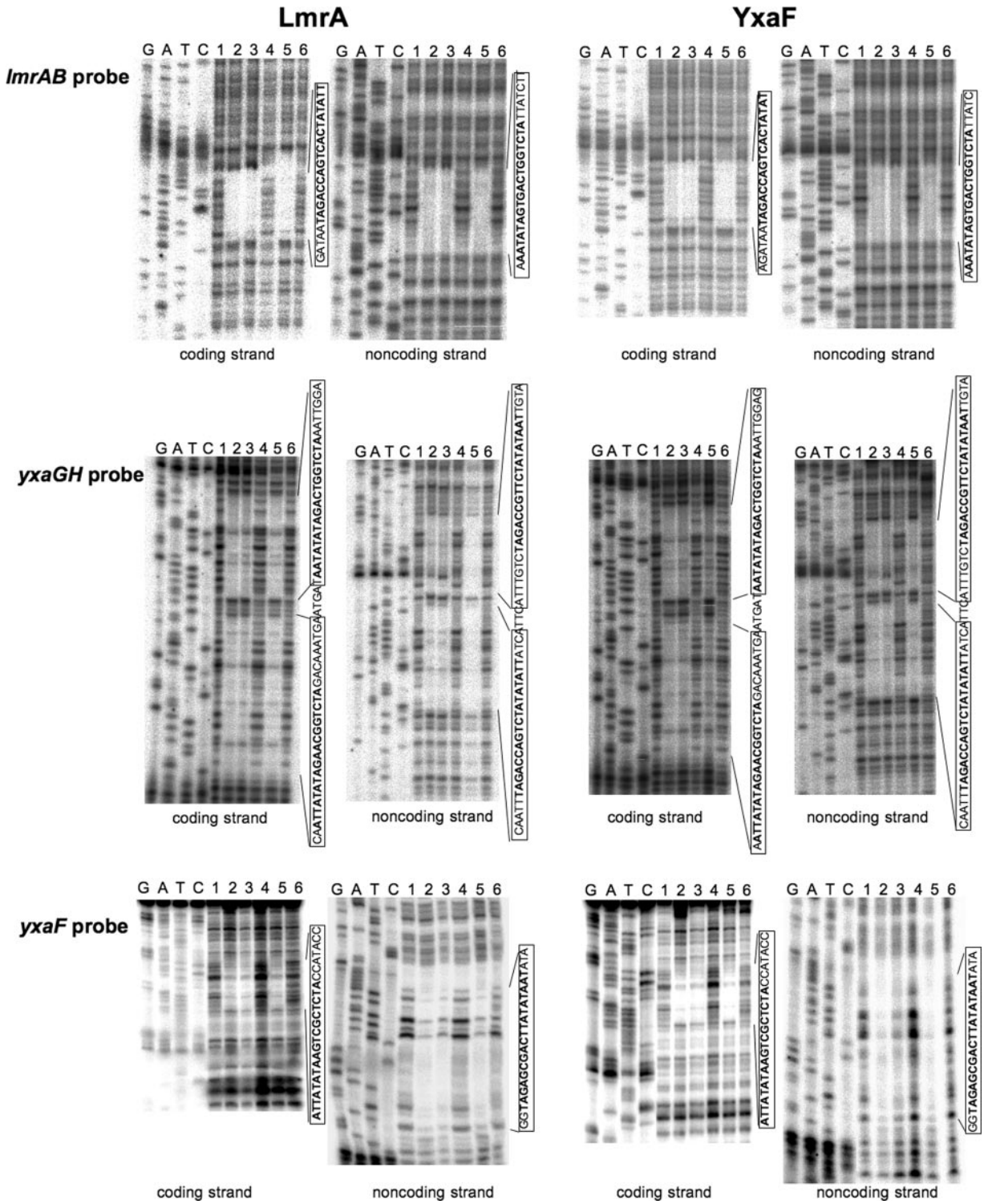


FIG. 4. DNase I footprinting of LmrA and YxaF in the *lmrAB*, *yxaGH*, and *yxaF* promoter regions. DNA probes corresponding to each of the promoter regions, 5′-end labeled at either the coding or noncoding strand, were prepared as described in Materials and Methods. The 5′-labeled probe (0.8 nM) was incubated in the reaction mixture with crude extract containing LmrA or YxaF protein (lanes 2, 5, and 6, 15 μg; lane 3, 7.5 μg), crude extract containing no repressor (lane 4, 15 μg), or no crude extract (lane 1). A quercetin solution in DMSO (2 μl) was added to the mixture in lane 6 to give 1.5 mM, and the same volume of DMSO was added to the mixture in lane 5 before incubation. After partial digestion with DNase I, the resulting mixtures were subjected to urea-PAGE. Lanes G, A, T, and C contain the products of the dideoxy sequencing reactions with the corresponding 5′-labeled primers. Nucleotide sequences protected by LmrA or YxaF are indicated in boxes on the right of each panel. The sequences of the regions protected against DNase I are shown in boldface letters.

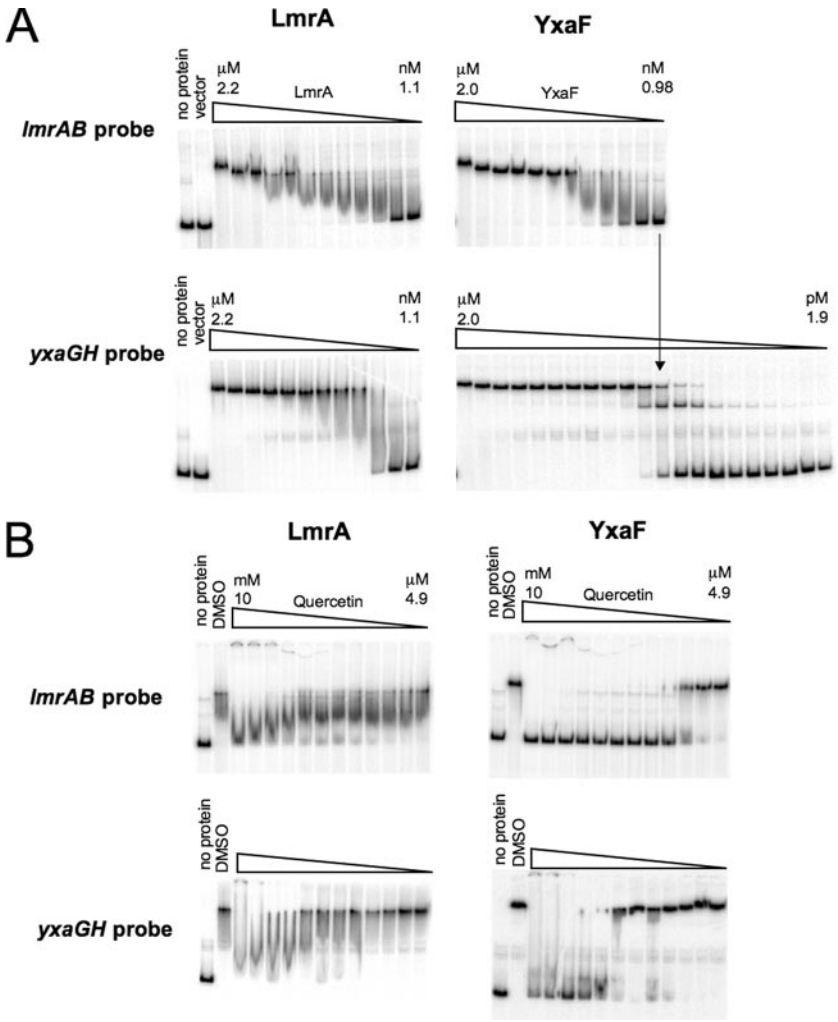


FIG. 5. Binding affinity of LmrA and YxaF for the *lmrAB* and *yxaGH* promoter regions, and the inhibitory effect of quercetin on it, as evaluated by gel retardation analysis. (A) The ^{32}P-labeled *lmrAB* and *yxaGH* probes (0.8 nM) were incubated with the LmrA and YxaF extracts, respectively, as described in Materials and Methods. The LmrA and YxaF extracts were diluted stepwise by twofold, and an aliquot of each dilution was added to the mixture. From the molecular masses of the LmrA and YxaF monomers (20,662 and 21,079 Da), 6 μg total protein of the extracts, containing 38 and 35% LmrA and YxaF, in the mixtures (25 μl) was calculated to correspond to 2.2 and 2.0 μM of the LmrA and YxaF dimers, respectively. The final concentrations of LmrA and YxaF in the mixtures were 2.2 μM to 1.1 nM (12 lanes, twofold dilution 11 times), 2.0 μM to 0.98 nM (12 lanes, twofold dilution 11 times), and 2.0 μM to 1.9 pM (21 lanes, twofold dilution 20 times), respectively. Lanes labeled "vector" contain 6 μg of total protein in the crude extracts prepared from *E. coli* BL21(DE3) bearing vector pET-22b(+), and lanes labeled "no protein" contain no protein, that is, no crude extract. Each of the experiments was repeated two times, and representative ones are shown. (B) Inhibitory effect of quercetin on the binding of LmrA or YxaF to the *lmrAB* and *yxaGH* promoter regions. The ^{32}P-labeled *lmrAB* and *yxaGH* probes (0.8 nM) were incubated with 138 nM LmrA or 125 nM YxaF in the presence of 4.9 μM to 10 mM quercetin (12 lanes, twofold dilution 11 times with DMSO), with DMSO being added to each of the mixtures. Lanes labeled "DMSO" contain only DMSO, and lanes labeled "no protein" contain no protein, that is, no crude extract. Each of the experiments was repeated at least two times, and representative ones are shown.

trol of the *spac* promoter, which is repressed by *E. coli* LacI (25). To monitor *lacZ* expression under the control of either the *lmrAB* or *yxaGH* promoter in the presence of LmrA or YxaF, we constructed a series of plasmid pMUTIN2 integrants of the *lmrA*, *lmrB*, *yxaG*, or *yxaF* gene, which carry either a *yxaF* or an *lmrA* disruption (Table 1). Strains FU878 (*lmrB*::pMUTIN2 Δ*yxaF*::*cat*) and FU879 (*yxaG*::pMUTIN2 Δ*yxaF*::*cat*) were used to monitor the *lmrAB* and *yxaGH* promoters, respectively, controlled by only LmrA. When quercetin was added to the culture in the logarithmic growth phase, a clear induction of β-Gal synthesis was observed in these strain cells, suggesting that quercetin was incorporated into the cells and functioned as an inducer to release the repression of the two promoters mediated only by LmrA (Fig. 6).

The use of strain LMRAd (*lmrA*::pMUTIN2) enabled us to examine if the *lmrAB* promoter is controlled only by YxaF. The basal β-Gal activity of strain LMRAd (8.7 nmol/min per OD) was considerably higher than that of strain FU878 (*lmrB*::pMUTIN Δ*yxaF*::*cat*) (3.2 nmol/min per OD), suggesting that the *lmrAB* promoter is repressed mainly by LmrA and not YxaF, and the promoter was further derepressed by quercetin (Fig. 6). (The basal β-Gal activity of strain LMRBd [Δ*lmrB*::pMUTIN] possessing both LmrA and YxaF was 1.48 nmol/min per OD [data not shown].) The *yxaGH* promoter controlled only by YxaF was mon-

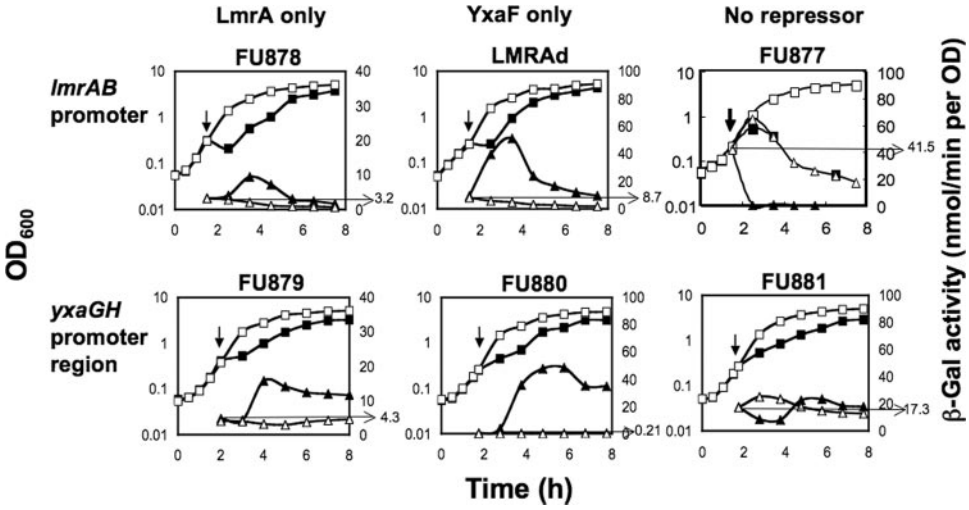


FIG. 6. Quercetin suppression of the *lmrAB* and *yxaGH* promoters repressed by LmrA or YxaF. Strains FU878 (Δ*yxaF*::*cat lmrB*::pMUTIN2) and FU879 (Δ*yxaF*::*cat yxaG*::pMUTIN2), strains LMRAd (*lmrA*::pMUTIN2) and FU880 (Δ*lmrA*::*tet yxaG*::pMUTIN2), and strains FU877 (Δ*yxaF*::*cat lmrA*::pMUTIN2) and FU881 (Δ*lmrA*::*tet* Δ*yxaF*::*cat yxaG*::pMUTIN2) possess only LmrA, only YxaF, and no repressor, respectively. Strains FU878, LMRAd, and FU877 and strains FU879, FU880, and FU881 were used to monitor the *lmrAB* and *yxaGH* promoter activities, respectively. Quercetin was added to the culture (as indicated by arrows), and the β-Gal activity was then monitored as described in Materials and Methods. The squares and triangles denote the OD_{600} values (squares) and the β-Gal activities (triangles) with (closed symbols) and without (open symbols) the addition of quercetin, respectively. The basal β-Gal activities before the addition of quercetin in the monitoring experiments are shown on the right of each panel (indicated by arrows). Each of the *lacZ* fusion experiments was repeated at least two times, and representative ones are shown.

itored by using strain FU880 (*yxaG*::pMUTIN2 Δ*lmrA*::*tet*), which indicated that quercetin derepressed it in a manner similar to that in the case of the *lmrAB* promoter. The basal activity of strain FU880 without the addition of quercetin (0.21 nmol/min per OD) was lower than that of strain FU879 (*yxaG*::pMUTIN2 Δ*yxaF*::*cat*) (4.3 nmol/min per OD), suggesting that YxaF might bind to the two LmrA/YxaF boxes in the *yxaGH* promoter region more tightly than LmrA, causing strict repression of the promoter. (The basal β-Gal activity of strain YXAGd [*yxaG*::pMUTIN] possessing LmrA and YxaF was 0.12 nmol/min per OD [data not shown].)

Although the promoter activities enhanced by quercetin gradually declined upon the further cultivation of strains FU878 (Δ*yxaF*::*cat lmrB*::pMUTIN2) and LMRAd (*lmrA*::pMUTIN2), the activities were sustained during the cultivation of strains FU879 (Δ*yxaF*::*cat yxaG*::pMUTIN2) and FU880 (Δ*lmrA*::*tet yxaG*::pMUTIN2) (Fig. 6). This observation suggests that the *yxaG* disruption in the latter strains likely prohibits quercetin degradation, leading to the continuous derepression of LmrA and YxaF by quercetin.

Strains FU877 (*lmrA*::pMUTIN2 Δ*yxaF*::*cat*) and FU881 (*yxaG*::pMUTIN2 Δ*lmrA*:*tet* Δ*yxaF*::*cat*) were constructed to monitor the activities of the *lmrAB* and *yxaGH* promoters in the absence of both LmrA and YaxF, respectively. Figure 6 shows that the β-Gal activities of strains FU877 (41.5 nmol/min per OD) and FU881 (17.3 nmol/min per OD) were not further enhanced upon the addition of quercetin. (The constitutive expression of *yxaGH* in strain FU881 was three times lower than its quercetin-triggered maximal expression in strain FU880, which is not explained properly at present.) Interestingly, the addition of quercetin provoked dramatic growth inhibition of only strain FU877 (Fig. 6); the growth of strain FU881 was not seriously affected by the addition of quercetin. From these results, we considered that the addition of quercetin might be harmful to the cells in which either or both of the *yxaG* and *yxaH* genes, encoding quercetin 2,3-dioxygenase and a putative transporter, respectively, are completely derepressed. To determine if *yxaH* is involved in this growth inhibition, *yxaH* expression under the control of the IPTG-inducible *spac* promoter in strain YXAGd (*yxaG*::pMUTIN2) was induced by the addition of 5 mM IPTG. It was found that the induced expression of the *yxaH* gene did not affect cell growth even when quercetin was added to the medium (data not shown). Moreover, the addition of quercetin also provoked dramatic growth inhibition of strain FU967 (Δ*lmrA*::*tet* Δ*yxaF*::*cat yxaH*::pMUTIN2), as observed with strain FU877 (*lmrA*::pMUTIN2 Δ*yxaF*::*cat*) (data not shown). These results suggest that the derepression of the synthesis of quercetin 2,3-dioxygenase encoded by *yxaG* synthesis is likely the reason for the growth defect in the presence of quercetin. This might be due to the accumulation of an unidentified intermediate(s) derived through rapid quercetin degradation by excess 2,3-dioxygenase, which is harmful to *B. subtilis*.

Screening of candidate flavonoids besides quercetin that derepress the control of LmrA and YxaF repressors. The fact that quercetin can interact with the LmrA and YxaF repressors to derepress the *lmrAB* and *yxaGH* promoters prompted us to investigate if other flavonoids could interact with them similarly. We devised a screening method for finding compounds such as flavonoids that inhibit the LmrA and YxaF repressors. We used strain YXAGd (*yxaG*::pMUTIN2) to monitor β-Gal synthesis reflecting *yxaGH* expression under the control of both LmrA and YxaF. If a tested flavonoid interacts with these regulatory proteins to dissociate them from the promoter, blue coloration would be produced around paper disks containing the flavonoid on a cell lawn on a TBABG plate containing

5-bromo-4-chloro-3-indolyl-β-D-galactoside. When 13 flavonoid compounds including quercetin were tested, quercetin, fisetin, tamarixetin, genistein, and coumestrol gave clear positive results, indicated by blue coloration around the disks, but 3-hydroxyflavone, galangin, robinetin, myricetin, morin, kaempferol, (+)-catechin, and daidzein did not (data not shown). We selected the newly found four positive compounds as candidate inducers and galangin, (+)-catechin, and daidzein as negative compounds, which were found with this screening system involving strain YXAGd, for further detailed in vitro and in vivo analyses to examine their interactions with LmrA and YxaF repressors.

Inhibitory effects of various flavonoids on the in vitro DNA binding of LmrA and YxaF. To determine whether the above-mentioned seven flavonoids besides quercetin can interact with LmrA and YxaF to specifically detach them from DNA, we carried out gel retardation analyses involving each of the flavonoids similarly to methods described above, the results of which are summarized in Table 3. When the *lmrAB* probe was incubated with LmrA in the presence of each of the flavonoids, clear inhibition of the DNA binding was observed with quercetin, fisetin, and (+)-catechin, whereas significant inhibition was observed with galangin, genistein, and coumestrol but not with tamarixetin and daidzein (Table 3). When binding analysis involving the *lmrAB* probe and the YxaF protein was conducted, clear inhibition was observed with flavonols (quercetin, fisetin, tamarixetin, and galangin), whereas significant inhibition was observed with genistein and coumestrol but not with (+)-catechin and daidzein.

On the other hand, when the *yxaGH* probe carrying two tandem LmrA/YxaF boxes was incubated with LmrA in the presence of each of the flavonoids, the DNA binding was inhibited by quercetin, fisetin, and (+)-catechin, as was LmrA binding to the *lmrAB* probe (Table 3). When the *yxaGH* probe and YxaF were used for the analysis, only quercetin and fisetin clearly inhibited DNA binding (Table 3), although they were less inhibitory to *yxaGH* binding of YxaF than to *lmrAB* binding of YxaF (Fig. 5B and Table 3). These results indicated that each of the flavonoids interacting with the repressors distinctly inhibited the DNA binding depending on not only the properties of the involved regulatory protein but also the sequence and/or tandem nature of the LmrA/YxaF box(es).

Suppressive effects of various flavonoids on the repression of the *lmrAB* and *yxaGH* promoters by LmrA and YxaF in vivo. Since each of the seven flavonoids besides quercetin was found to inhibit the binding of LmrA and YxaF to the LmrA/YxaF boxes in vitro in different manners, we examined, through *lacZ* fusion experiments, if they actually release the repression of the *lmrAB* and *yxaGH* promoters through the LmrA and YxaF repressors, depending on the repressor or LmrA/YxaF box involved, as observed by in vitro gel retardation analysis.

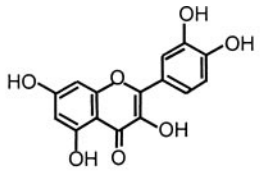
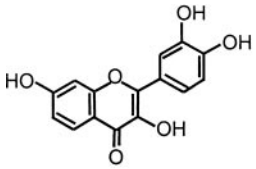
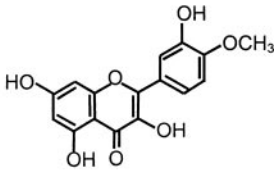
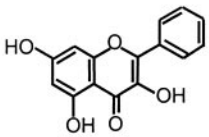
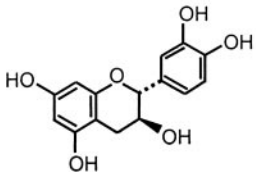
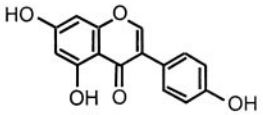
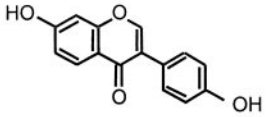
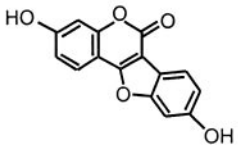
The results are summarized in Table 3, together with the those obtained from the in vitro DNA binding assay. The *lmrAB* promoter activity controlled by only LmrA was monitored using strain FU878 (Δ*yxaF*::*cat lmrB*::pMUTIN2). When quercetin, fisetin, genistein, or coumestrol was added to the medium, the β-Gal activity increased significantly, with fisetin being the most effective (Table 3). The addition of (+)-catechin caused slight but continuous β-Gal synthesis (data not shown), but tamarixetin, galangin, and daidzein hardly induced it (Table 3). The results indicate that the five flavonoids that enhance β-Gal synthesis are able to be incorporated into the cells and to function as inducers to release LmrA from the promoter. Furthermore, the derepression of the *lmrAB* promoter repressed by YxaF was examined using strain LMRAd (*lmrA*::pMUTIN2) (Table 3). The β-Gal activity was well induced not only by quercetin, fisetin, genistein, and coumestrol but also by tamarixetin and daidzein; the latter did not suppress the *lmrAB* repression by LmrA. However, galangin and (+)-catechin did not release this repression. These in vivo results essentially agreed with those obtained using the in vitro gel retardation analysis, except that only galangin did not suppress the repression of *lmrAB* through LmrA or YxaF.

The *yxaGH* promoter activity controlled by LmrA only was monitored by measuring β-Gal synthesis in strain FU879 (Δ*yxaF*::*cat yxaG*::pMUTIN2). When quercetin, fisetin, genistein, or coumestrol was added to the culture, the β-Gal activity increased substantially, with fisetin being the most effective. The addition of (+)-catechin enhanced it slightly, and the elevated activity was sustained during cultivation (data not shown). These results coincided well with the results of the corresponding in vitro experiments, except that galangin did not release the repression in vivo. On the other hand, the activity of the *yxaGH* promoter repressed only by YxaF was monitored by using strain FU880 (Δ*lmrA*::*tet yxaG*::pMUTIN2); the addition of quercetin, fisetin, or genistein induced β-Gal synthesis significantly, which is consistent with the results of the in vitro analysis. However, in conflict with the in vitro results, tamarixetin and coumestrol also clearly induced β-Gal synthesis (Table 3) The probable reasons for this contradiction are discussed below. Galangin did not induce β-Gal synthesis in this case, either. The fact that galangin did not induce β-Gal synthesis in any case, contrary to the in vitro results, suggests that galangin is unable to be incorporated into *B. subtilis* cells.

The β-Gal syntheses reflecting the respective *lmrAB* and *yxaGH* promoter activities in strains FU877 (Δ*yxaF*::*cat lmrA*::pMUTIN2) and FU881 (Δ*lmrA*::*tet* Δ*yxaF*::*cat yxaG*::pMUTIN2), defective in both LmrA and YxaF, were constitutive, regardless of the addition of each of the flavonoids. However, the severe growth defect of strain FU877 observed with quercetin (Fig. 6) did not occur with any of the other flavonoids (data not shown).

Dual regulation of MDR and quercetin 2,3-dioxygenase synthesis by LmrA and YxaF. Previous studies showed that the inactivation of the LmrA protein caused the constitutive expression of *lmrB* to elevate resistance to drugs such as lincomycin and puromycin (14, 30). The results obtained in this work revealed that in addition to LmrA, YxaF also binds to the *lmrAB* promoter region to repress *lmrAB* expression. Thus, to determine whether YxaF also regulates this MDR reflecting *lmrB* expression, we measured the growth rate in LB medium containing various concentrations of lincomycin and determined IC_{50}s of *B. subtilis* strains in which either or both LmrA and YxaF were inactivated (Table 4). Strain PLR2, in which mutant LmrA with two point mutations is unable to repress LmrB synthesis, showed higher lincomycin resistance than strain 168 (Table 4), as reported previously (30). On the other hand, strain FU875 (Δ*yxaF*::*cat*) exhibited almost the same sensitivity to lincomycin as strain 168. The introduction of the *yxaF* disruption in a PLR2 background to the constructed strain FU899 caused a slight increase in lincomycin resistance

TABLE 3. Structures of flavonoids, their inhibitory effects on LmrA and YxaF binding to the LmrA/YxaF boxes, and derepression of the *lmrAB* and *yxaGH* promoters repressed by them

Flavonoid	Structure[a]	Effect in vitro (K_i [mM])/effect in vivo on protein with indicated promoter[b]			
		LmrA		YxaF	
		lmrAB	*yxaGH*	*lmrAB*	*yxaGH*
Quercetin[c]		+++ (0.8)/++	+++ (1.0)/++	++++ (0.05)/+++	++++ (0.47)/+++
Fisetin		++++ (0.23)/+++	++++ (0.94)/+++	++++ (0.08)/+++	++++ (0.63)/+++
Tamarixetin		−/−	−/−	++++ (0.02)/+++	−/+++
Galangin		++ (5.0)/−	++ (5.0)/−	++++ (0.31)/−	+/−
Catechin		++++ (0.63)/+	++++ (0.31)/+	−/−	−/−
Genistein		++ (5.0)/++	++ (7.5)/++	++ (1.3)/++	+/++
Daidzein		+/−	−/−	+/++	−/−
Coumestrol		++ (2.5)/++	+/++	++ (2.5)/+++	−/+++

[a] The structure of flavone is presented at the top of the column.

[b] Data are presented as an evaluation of the in vitro results compared to that of the in vivo results. The in vitro results were obtained through experiments involving gel retardation, which were performed as described in the legend of Fig. 5B, where those with the addition of quercetin are shown. According to the K_i values for the eight flavonoids regarding the binding of LmrA and YxaF to the *lmrAB* and *yxaGH* probes, the inhibitory effects are classified as follows: ++++, <1.0 mM; +++, 1.0 to 2.5 mM; ++, 2.5 to 10 mM; +, >10 mM; −, no inhibition. The actual K_i values are given for the combinations of LmrA/YxaF repressors and boxes belonging to the categories ++++, +++, and ++. The parenthetical K_i values are given in mM. The in vivo results were obtained through experiments involving *lacZ* fusions, which were performed as described in the legend of Fig. 6, where those with the addition of quercetin are shown. According to the peak β-Gal activity after the addition of each flavonoid, the derepression degrees of the *lmrAB* and *yxaGH* promoters repressed by LmrA and YxaF are graded as follows: +++, >20 nmol/min per OD; ++, 10 to 20 nmol/min per OD; +, <10 nmol/min per OD; −, no derepression.

[c] The data for the quercetin effect are those shown in Fig. 5B and 6.

TABLE 4. Dual regulation of lincomycin resistance and quercetin 2,3-dioxygenase synthesis by LmrA and YxaF

Strain	Disrupted gene(s)	Mean lincomycin IC_{50} (μg/ml) ± SD[a]	Quercetin 2,3-dioxygenase concn (μmol/min per mg protein) ± SD[a]
168	None	14.6 ± 5.0	0.0004 ± 0.0002
FU875	*yxaF*	15.6 ± 5.1	0.0368 ± 0.0126
PLR2	*lmrA*	170.5 ± 55.9	0.145 ± 0.024
FU899	*lmrA* and *yxaF*	196.4 ± 53.9	0.568 ± 0.136

[a] Data represent the mean values ± standard deviations obtained in three independent experiments.

in comparison with that of strain PLR2 (Table 4). These results indicate that LmrA regulates *lmrAB* expression predominantly, whereas YxaF contributes to its regulation accessorily.

We also examined how LmrA or YxaF controls the activity of quercetin 2,3-dioxygenase, encoded by *yxaG*. Cells of strains 168, PLR2, FU875, and FU899 were cultivated and harvested, crude extracts were prepared, and the quercetin 2,3-dioxygenase activities in the extracts were determined spectrophotometrically (Table 4). The cell extract of strain FU875 (Δ*yxaF*::*cat*) exhibited considerably high enzymatic activity in comparison with that of strain 168, whereas the extract of the *lmrA* mutant strain PLR2 exhibited higher activity than that of strain FU875. The enzyme activity in the extract of the *lmrA* and *yxaF* doubly mutated strain FU899 was the highest among those of the four strains. The results indicate that both LmrA and YxaF repress *yxaGH* expression, although LmrA contributes more to its regulation than YxaF does. Moreover, YxaF likely represses the *yxaGH* promoter more preferentially than the *lmrAB* promoter. When the cells of the above-described four strains were exposed to quercetin for 2 h under the cultivation conditions adopted for the *lacZ* fusion experiments (Fig. 6), only the quercetin 2,3-dioxygenase activity in the lysate obtained from strain 168 was accurately determined due to the harmful effect of quercetin on the mutant cells, which was 0.860 ± 0.182 μmol/min per mg protein. This value is higher than the derepressed activity of strain FU899 (0.568 ± 0.136 μmol/min per mg), indicating that quercetin is able to completely inhibit LmrA and YxaF in vivo.

DISCUSSION

The LmrA and YxaF proteins belong to the TetR family of bacterial DNA binding transcriptional regulatory proteins, which exhibit 56% identity in their amino acid sequences, with their N-terminal regions including the HTH motif for DNA binding being especially conserved (Fig. 2). The DNase I footprinting analysis revealed that both LmrA and YxaF specifically recognize and bind to the four LmrA/YxaF boxes containing an 18-bp consensus sequence, AWTATAtagaNYGgT CTA, which are located in the promoter regions of the *lmrAB* operon, the *yxaF* gene, and the *yxaGH* operon; the two tandem LmrA/YxaF boxes are positioned in the *yxaGH* promoter region. Thus, it is believed that the LmrA/YxaF regulon comprises the *lmrAB* operon, the *yxaF* gene, and the *yxaGH* operon. To search for additional members of the LmrA/YxaF regulon, we performed DNA microarray analysis using total RNAs derived from cells of strains 168 (wild type) and FU877 (Δ*yxaF*::*cat lmrA*::pMUTIN2) deficient in LmrA and YxaF, which were grown to the mid-logarithmic phase in LB medium (data not shown). However, we were able to find no other candidate target genes for LmrA/YxaF regulon members, which are presumably repressed by both LmrA and YxaF, upon comparisons of the two transcriptome patterns, suggesting that the members of the LmrA/YxaF regulon might be only the above-described three genes/operons revealed in this study.

The TetR family of bacterial transcriptional regulatory proteins is known to typically possess two functional domains, a highly conserved N-terminal DNA binding domain and a less conserved C-terminal domain involved in both dimerization and effector binding (17). Recently, a crystal structure of the YxaF protein was determined (21). As predicted from the crystal structure, the YxaF protein possesses the HTH motif constituting the core of the putative DNA binding domain in the N-terminal region and forms the dimer structure in which the C-terminal domains are associated with each other. In this study, we found that the binding of LmrA and YxaF to the above-described four LmrA/YxaF boxes is inhibited by several flavonoids, such as quercetin and fisetin, resulting in the suppression of the *lmrAB*, *yxaF*, and *yxaGH* promoters repressed by them (Fig. 3 to 6 and Table 3). Thus, the effectors interacting with the C-terminal domains of LmrA and YxaF, i.e., inducers of the LmrA/YxaF regulon, are concluded to be the effective flavonoids shown in Table 3. Although LmrA and YxaF are quite similar to each other in function, they are distinct from each other in the following aspects. First, LmrA is less stable than YxaF; LmrA loses the DNA binding activity easily during the purification steps (data not shown). Second, the affinity of binding of YxaF to the LmrA/YxaF boxes is higher than that of LmrA; YxaF exhibits extremely high affinity, especially to the tandem boxes in the *yxaGH* promoter (Fig. 5A). The lower binding affinity of LmrA than that of YxaF (Fig. 5A) might be partially attributed to the instability of LmrA. Finally, the flavonoids that specifically inhibit LmrA binding to the LmrA/YxaF boxes are substantially different from those that inhibit YxaF binding to them (Table 3).

According to the results of the in vitro experiments (Fig. 5B and Table 3), LmrA binding to the *lmrAB* and *yxaGH* LmrA/YxaF boxes was well inhibited by quercetin, fisetin, and (+)-catechin, whereas it was less inhibited by galangin, genistein, and coumestrol and was inhibited hardly at all by tamarixetin and daidzein. When the inhibitory effects of the flavonols (quercetin, fisetin, tamarixetin, and galangin) on the DNA binding of LmrA are compared (Table 3), those of quercetin and fisetin can be seen to be higher than that of galangin, and tamarixatin has no inhibitory effect on it. These results suggest that the 4′-hydroxyl group at the B ring of flavonols (refer to the flavone structure for the ring and carbon assignments in Table 3) is essential for the DNA binding of LmrA but that the 5-hydroxyl group at the A ring is dispensable. Moreover, (+)-catechin is very inhibitory to the DNA binding of LmrA, suggesting that the plane structure of the center ring of the flavonoid might not be a determinant for its inhibitory effect. Isoflavones (genistein, daidzein, and coumestrol) exhibit less inhibitory effects than quercetin and fisetin, implying that the positioning of the B ring at the 3 carbon of the A ring is

improper for the inhibitory effect. Coumestrol was also less recognized by LmrA than quercetin and fisetin.

On the other hand, the in vitro experiments involving YxaF revealed that YxaF binding to the *lmrAB* probe is well inhibited by all the flavonols tested (quercetin, fisetin, tamarixetin, and galangin) but was less inhibited by the isoflavones tested (genistein, daidzein, and coumestrol) (Table 3). YxaF binding to the *yxaGH* probe carrying the tandem LmrA/YxaF boxes was tighter than that to the *lmrAB* probe; the binding and association constants were roughly 5 and 1 nM, respectively (Fig. 5A). Moreover, this tight binding of YxaF to the *yxaGH* probe was less inhibited by quercetin (Fig. 5B) and fisetin (data not shown) than that to the *lmrAB* probe; the K_i values of quercetin were approximately 0.05 mM and 0.5 mM for YxaF binding to the *lmrAB* and *yxaGH* probes, respectively. Smaller K_i values for YxaF binding to the *lmrAB* probe were also obtained with the other flavonoids (fisetin, tamarixetin, galangin, genistein, and coumestrol) (Table 3). In contrast to LmrA binding to the LmrA/YxaF boxes, YxaF binding was not inhibited by (+)-catechin, suggesting that the plane structure of the flavonol central ring might be indispensable for its inhibition effect.

The in vivo experiments involving the *lacZ* reporter gene showed that several flavonoids were able to induce the expression of the reporter gene placed downstream of the *lmrAB* or *yxaGH* promoter in the presence of either LmrA or YxaF, which well supports the in vitro results described above, except that galangin did not induce the β-Gal activity in any case, probably due to insufficient incorporation of galangin into the cells, and except that in contrast to the in vitro results, the repression of *yxaGH* by YxaF is well released by effective flavonoids in vivo, such as tamarixetin and coumestrol, as observed with that of *lmrAB* (Table 3). The latter contradiction might be possibly attributed to the possibility that the concentration of YxaF used in the in vitro experiments (125 nM) is likely higher than that in vivo because *yxaF* expression was very low as observed with the *lacZ* fusion experiments (data not shown); a lower concentration of YxaF might be sufficient to cause the repression of the *lmrAB* and *yxaGH* promoters under the physiological in vivo conditions, which could be easily relieved by the effective flavonoids.

As shown in Fig. 4, the LmrA and YxaF repressors recognize and bind to the LmrA/YxaF boxes of the LmrA/YxaF regulon. Thus, the physiological significance of the dual repression system through LmrA and YxaF for the *lmrAB* and *yxaGH* operons should be addressed, focusing on the predominance of either LmrA or YxaF for the repression of the *lmrAB* and *yxaGH* promoters. As shown in Fig. 5A, YxaF exhibited the highest affinity to the *yxaGH* probe, although it is not clear to which probe LmrA exhibited higher affinity. The in vivo results show that the *lmrAB* promoter was relatively more repressed by LmrA than by YxaF, whereas the *yxaGH* promoter was more tightly repressed by YxaF than by LmrA (refer to the basal β-Gal activities in Fig. 6). On the other hand, the lincomycin resistance reflecting *lmrB* expression was predominantly repressed by LmrA and was repressed by YxaF accessorily (Table 4). Furthermore, quercetin 2,3-dioxygenase synthesis reflecting *yxaG* expression was repressed well by YxaF and more by LmrA. From these in vivo results, it is concluded that LmrA represses both the *lmrAB* and *yxaGH* promoters but that YxaF represses the *yxaGH* promoter more preferentially.

The dual regulation of *yxaGH* expression through LmrA and YxaF might be related to the degradation of toxic flavonoids as follows. Some flavonoids are known to possess antibacterial activity (24). Out of the eight flavonoids tested for the inhibitory effect on the DNA binding of LmrA and YxaF, quercetin exhibited the most significant inhibitory effect on the growth of *B. subtilis* cells when each flavonoid was added at a concentration of 200 μg/ml (Fig. 6). This inhibitory effect of quercetin was most pronounced when it was added to the culture of strain FU877 (Δ*yxaF*::*cat lmrA*::pMUTIN2), in which *yxaGH* expression was completely derepressed in the absence of LmrA and YxaF. This severe growth-inhibitory effect of quercetin was most likely due to the derepression of *yxaG*, encoding quercetin 2,3-dioxygenase, because the induction and disruption of *yxaH* did not enhance it, suggesting that the rapid accumulation of a harmful degradation intermediate(s) of quercetin might damage the mutant cells, resulting in growth inhibition. This fact might be related to the complicated dual regulation of *yxaGH* expression through LmrA and YxaF, as described above, because cells must rigorously regulate 2,3-dioxygenase activity toward a wide range of concentrations of toxic flavonoids such as quercetin.

The LmrA/YxaF regulon characterized in this work is the first system in the *Bacillus* genus in which flavonoids induce a regulon through the inactivation of LmrA and YxaF. Some other bacterial transcriptional regulators that recognize and respond to flavonoid compounds were reported previously. NodD regulators, which belong to the LysR family and control the transcription of the *nod* operons involved in the nodulation of *Rhizobiales* in response to flavonoid signals released by their leguminous hosts (9), have been characterized in detail. Moreover, in *Pseudomonas putida* DOT-T1E, the resistance-nodulation-cell division family transporter TtgABC and its cognate TetR family repressor TtgR both possess multidrug recognition properties, and several flavonoids are substrates of TtgABC and activate pump expression by binding to the TtgR-operator complex to dissociate it (23). Hence, it might not be rare that flavonoids function as signaling molecules for communication among soil bacteria and plants. However, our attempt to find a conserved motif for flavonoid recognition by aligning the amino acid sequences of these regulators was unsuccessful.

An interesting question arises regarding why some unique flavonoids participate in the resistance to structurally unrelated drugs such as lincomycin via the LmrA/YxaF regulation system in *B. subtilis*. All of the *lmrAB*-related MDR mutants isolated so far possess mutations in the LmrA/YxaF binding site of the *lmrAB* promoter and/or the *lmrA* gene, which causes *lmrB* overexpression (12, 14), and lincomycin itself does not act as an inducer molecule for LmrA (30) and YxaF (data not shown). Nonetheless, this puzzle remains to be elucidated. It is also unclear whether the degradation of quercetin to 2-protocatechuoyl-phloroglucinol carboxylic acid by the YxaG protein is aimed at detoxification or at dissimilation to utilize it as a carbon source. The *yxaH* gene encodes a putative membrane protein with nine transmembrane segments. Although its physiological function remains unknown, the YxaH membrane protein might contribute to

either the degradation or efflux of related flavonoids. It would be intriguing to reveal the overall metabolic pathway for flavonoids in *B. subtilis*.

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