

Bacillus subtilis LmrA Is a Repressor of the *lmrAB* and *yxaGH* Operons: Identification of Its Binding Site and Functional Analysis of *lmrB* and *yxaGH*

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The *Bacillus subtilis* *lmrAB* operon is involved in multidrug resistance. LmrA is a repressor of its own operon, while LmrB acts as a multidrug efflux transporter. LmrA was produced in *Escherichia coli* cells and was shown to bind to the *lmr* promoter region, in which an LmrA-binding site was identified. Genome-wide screening involving DNA microarray analysis allowed us to conclude that LmrA also repressed *yxaGH*, which was not likely to contribute to the multidrug resistance. LmrA bound to a putative *yxaGH* promoter region, in which two tandem LmrA-binding sites were identified. The LmrA regulon was thus determined to comprise *lmrAB* and *yxaGH*. All three LmrA-binding sites contained an 18-bp consensus sequence, TAGACCRKTCWMTATA WT, which could play an important role in LmrA binding.

Multidrug resistance of bacteria is one of the most severe problems in the clinical treatment of infectious diseases. To date, it has been shown that many of the mechanisms for bacterial multidrug resistance require the expression of genes for drug efflux transporters (1, 12). In *Bacillus subtilis*, at least three genes encoding multidrug resistance efflux transporters have been characterized, namely, *blt*, *bmr*, and *bmr3* (2, 14, 16). It was shown previously that *bmr* and *blt* were induced in the presence of their substrates under control of the transcriptional regulators BmrR and BltR, respectively (21, 28). Both these regulators belong to the MerR family of activators, and at least DNA binding of BmrR required interaction with a Bmr substrate, such as rhodamine 6G or tetraphenylphosphonium, as a coactivator (21, 27, 28). Recently, *B. subtilis* *lmrB* was found to encode a fourth multidrug efflux transporter belonging to the major facilitator superfamily (13). The *lmrB* gene is the second gene of the *lmrAB* operon (Fig. 1A) (10), and the first gene, *lmrA*, was thought to encode a transcriptional repressor of the TetR family (3).

A mutation in the *lmr* promoter region (*lin-2*) that confers lincomycin resistance in *B. subtilis* 1A221 through enhanced expression of *lmrAB* has been reported (7). Additionally, 19 spontaneous lincomycin-resistant mutants have been isolated and have also been shown to elevate the expression of *lmrAB* (11). Eighteen of these 19 mutants had mutations in the promoter region with no alternation in the *lmrA* coding region, while the remaining mutant possessed no mutation in the promoter region but had a substitution in the *lmrA* termination codon, which extended the C terminus for 9 amino acid resi-

dues [*lmrA*(stop to S)] (11, 13). In a subsequent study, two mutants selected in the presence of high concentrations of lincomycin and puromycin, PLR1 and PLR2, were isolated and shown to exhibit multidrug resistance (13). Both PLR1 and PLR2 had increased expression of *lmrAB*, and inactivation of *lmrB* in each of them abolished the multidrug resistance. PLR1 had a nucleotide substitution in the *lmr* promoter region, while PLR2 possessed no mutation in the promoter region but had two mutations in the *lmrA* coding region, *lmrA*(stop to S) and *lmrA*(Q52P) within the putative helix-turn-helix motif of the TetR family found between the 10th and 57th amino acid residues (BSORF website [<http://bacillus.genome.ad.jp/>]). These findings implied that LmrA might be a repressor that interacts with the promoter region of its own operon.

In this study we found that LmrA is a repressor of the *lmrAB* operon, and we identified its binding site in the *lmr* promoter region. During systematic genome-wide screening, an additional LmrA target, *yxaGH*, was identified, and in its putative promoter region two tandem LmrA-binding sites were found.

MATERIALS AND METHODS

Bacterial strains and plasmids. *B. subtilis* strain 168 (*trpC2*) was used as the wild-type strain. *B. subtilis* strain 1A221 (*lin-2*) (7), a lincomycin-resistant mutant, was obtained from the *Bacillus* Genetic Stock Center (Columbus, Ohio). *B. subtilis* strains PLR1 [*trpC2* *Plmr*(A-1T) *lmrA*(stop to S)], PLR2 [*trpC2* *lmrA*(Q52P stop to S)], and PLR3 (= PLR2/*lmrB*::*cat*) [*trpC2* *lmrA*(Q52P stop to S) *lmrB*::*cat*] were produced in a previous study (13). *B. subtilis* strains YXAGd (*trpC2* *yxaG*::pMUTIN2) and YXAHd (*trpC2* *yxaH*::pMUTIN2) are two of the pMUTIN2 integrants (20) of strain 168 constructed in the course of a functional analysis in the *B. subtilis* genome project (24) (BSORF website). Strains PLR2 and PLR3 were transformed with DNA from YXAGd, and this was followed by selection for erythromycin resistance, which yielded strains PLR4 [*trpC2* *lmrA*(Q52P stop to S) *yxaG*::pMUTIN2] and PLR5 [*trpC2* *lmrA*(Q52P stop to S) *lmrB*::*cat* *yxaG*::pMUTIN2], respectively. Strains PLR6 [*trpC2* *lmrA*(Q52P stop to S) *yxaH*::pMUTIN2] and PLR7 [*trpC2* *lmrA*(Q52P stop to S) *lmrB*::*cat* *yxaH*::pMUTIN2] were constructed as described above by using DNA of YXAHd. *Escherichia coli* strain JM109 and plasmid pUC18 (22) were used to

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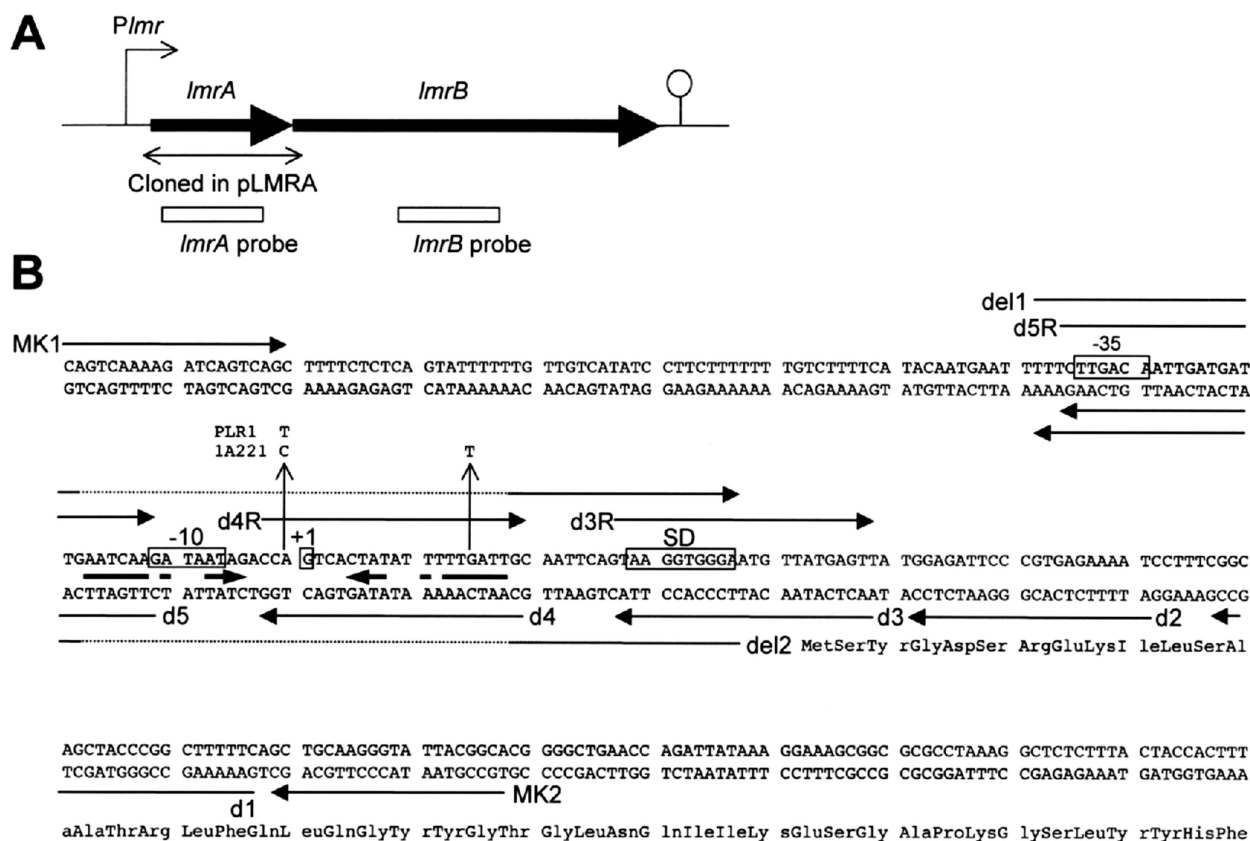


FIG. 1. Organization of the *lmrAB* operon and its promoter region. (A) Organization of the *lmrAB* operon. The regions cloned into plasmid pLMRA for LmrA production in *E. coli* and used as probes for the Northern analysis are indicated. (B) *lmr* promoter region. The sequences of the DNA strands corresponding to the *lmr* promoter region and the N-terminal part of the *lmrA* coding region are shown (the amino acid sequence of the coding region is indicated beneath the nucleotide sequence). The positions and orientations of PCR primers used to prepare gel retardation probes are indicated by thin horizontal arrows. The dotted lines in the arrows for the del1 and del2 primers indicate the internally deleted 36-bp stretch. The -10 and -35 regions, the transcription start site (position 1) of the promoter, and the Shine-Dalgarno sequence (SD) are enclosed in boxes. The pair of discontinuous thick horizontal arrows facing each other indicates an incomplete palindrome sequence. The vertical arrows indicate the mutation points found in strains PLR1 and 1A221.

clone and express *lmrA* as described below. *B. subtilis* cells were grown on tryptose blood agar base (Difco) plates supplemented with 0.18% glucose at 30°C and in Luria-Bertani (LB) liquid medium (18) at 37°C with shaking. *E. coli* cells were grown on LB medium plates at 37°C and in TGA liquid medium (9) at 37°C with shaking. Ampicillin (50 µg/ml), erythromycin (0.3 µg/ml), and lincomycin (100 µg/ml) were added to culture media for selection and growth of mutants and transformants as required.

Production of LmrA in *E. coli* cells. For production of LmrA in *E. coli* cells, plasmid pLMRA was constructed as follows. A 0.6-kb PCR fragment, which contained the entire reading frame of *lmrA* with its corresponding ribosome binding site (Fig. 1A), was amplified from *B. subtilis* strain 168 genomic DNA by using a pair of primers, primers *lmrAE* and *lmrAB* (Table 1), which were designed to generate EcoRI and BamHI sites at the head and tail of the fragment, respectively. This fragment was trimmed with EcoRI and BamHI and then ligated with the arm of plasmid pUC18 that had been cleaved with the same enzymes. *E. coli* JM109 was transformed with the ligated DNA described above to obtain ampicillin-resistant colonies on LB plates. Plasmid DNA was extracted from one of the transformants, and its correct construction was confirmed by nucleotide sequencing in order to produce plasmid pLMRA. JM109 cells carrying pLMRA were grown in TGA medium containing 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to induce *lmrA* under control of the pUC18-borne *lac* promoter.

Gel retardation and DNase I footprinting experiments. Gel retardation experiments were performed as described previously (23). A protein extract was prepared from strain JM109 cells carrying either plasmid pLMRA or plasmid pUC18 grown in the presence of 1 mM IPTG. For analysis of the *lmr* promoter

region, DNA probes (Fig. 2A) were amplified and labeled by PCR in the presence of [α -³²P]dCTP (ICN Biomedicals) by using *B. subtilis* strain 168 genomic DNA as a template and specific primer pairs (Fig. 1B and Table 1). A probe carrying an internal deletion of a 36-bp region carrying the incomplete palindrome (Fig. 1B) was prepared as follows. A DNA fragment with the deletion was amplified by recombinant PCR (8) from DNA of strain 168 by using a flanking primer pair (primers MK1 and MK2) and an internal overlapping primer pair (primers del1 and del2) (Fig. 1B and Table 1). The correct deletion was confirmed by DNA sequencing. The fragment was used as a template for subsequent PCR in the presence of [α -³²P]dCTP by employing the pair of flanking primers described above to obtain a labeled probe with the internal deletion. To confirm the putative LmrA-binding sites predicted as described below, labeled probes designed to carry each of the putative sites were prepared. The PyxaG probe (see Fig. 5A), an example of such a probe, was a PCR fragment derived from strain 168 DNA that was amplified and labeled by using the specific primer pair PyxaG1 and PyxaG2 (Table 1). Each of the labeled probes (0.02 pmol) was combined with various amounts of *E. coli* protein extract in a reaction mixture (25 µl) (10 mM Tris-Cl [pH 7.6], 1 mM Na-EDTA, 0.1 mM dithiothreitol, 2 µg of bovine serum albumin per µl) in the presence of 3.3 µg of fragmented salmon sperm DNA as described previously (23), and then the mixture was subjected to 5% polyacrylamide gel electrophoresis. For the competition assay, specific competitors were synthesized in the form of PCR fragments amplified from DNA of strains 168, 1A221, and PLR1 with the MK1-MK2 primer pair. Various concentrations of these competitors were added to the assay mixture to compete for LmrA binding with a fixed amount of the labeled probe prepared from strain 168 DNA by using the same primer pair.

TABLE 1. PCR primers used in this study

Primer	Sequence (5'-3')
1mrAE	CCGGAATTCGATTGCAATTCAGTAAGG TGG (EcoRI site is underlined)
1mrAB	CGCGGATCCATGCTTTAGCTGTTGTTT CC (BamHI site is underlined)
MK1	CAGTCAAAGATCAGTCAGC
MK2	TGCCGTAATACCCTTGCAGC
d1	AAAAGCCGGGTAGCTGCC
d2	GATTTTCTCACGGGAATCTC
d3	AACTCATAACATTCCCACCTTA
d4	GCAATCAAAAATATGTGACTGG
d5	CTTGATTCAATCAATCATCAATTGTCA AG
d3R	TAAGGTGGGAATGTTATGAGTT
d4R	CCAGTCACATATTTTGATTGC
d5R	CTTGACAATTGATGATTGAATCAAG
de11	TTCTTGACAATTGATGATTGGCAATTC AGTAAGGTGGGAA
de12	TTCCACCTFACTGAATTGCCAATCAT CAATTGTCAAGAA
PyaG1	GGTGAGGAAAAAGGGTAGC
PyaG2	CTCCGGAGCAAATAAGGCAT
NlmrA1	ACGGGGCTGAACCAGATTAT
NlmrA2	CAGGAATGCAGCTGGAGATA
NlmrB1	GTATTCCGATTCAGCAACGC
NlmrB2	ATCATCAAGCAGGTGTGCAG
NyxaG1	TAGATGAAGCAAAGCCTGCA
NyxaG2	AAAGCGCAAAGCCTGAGGCT
NyxaH1	TGCGGCAGCAAAATCACGGT
NyxaH2	CAATAGGCTGCCACGACAGA

DNase I footprinting experiments were performed as described previously (23). The same protein extracts that were used in the gel retardation experiments described above were employed. The probes for DNase I footprinting experiments were prepared by PCR amplification of fragments of strain 168 DNA by using the specific primer pair MK1-MK2 (for the *lmr* promoter region) and PyaG1-PyaG2 (for the *ypaGH* promoter region). Prior to PCR amplification, the 5' termini of the primers were labeled by using a Megalabel kit (Takara Shuzo) with [γ - 32 P]ATP (Amersham) so that either the coding strand or the noncoding strand was labeled.

DNA microarray analysis and GRASP-DNA search for putative LmrA binding sites. To screen for additional LmrA targets, we employed a strategy analogous to that used in a previous study involving combined DNA microarray and gel retardation analyses (26). DNA microarray analysis was performed as described previously (26). *B. subtilis* strains PLR2 and 168 were grown in LB liquid medium, and then the cells were harvested in the middle of the logarithmic phase at an optical density at 600 nm of 0.5 and disrupted to extract total RNA by vigorous shaking with glass beads in the presence of sodium dodecyl sulfate and phenol (25). The two RNA samples were converted to cDNAs, and this was followed by differential labeling with a fluorescent dye, Cy3 or Cy5 (15). The differentially labeled cDNAs were mixed and hybridized to a glass slide microarray on which probe DNAs of 4,005 *B. subtilis* genes and control DNAs had been spotted (25). After washing, the microarray was scanned with a GMS 418 array scanner (Affymetrix/Genetic MicroSystems) to generate two images of dye-specific fluorescence. The signals in these images were quantified by using ImaGene software (version 4.1; Biodiscovery). Subsequently, the signal data set was processed as described previously (26) to identify genes exhibiting altered expression profiles, and then putative transcription units containing such genes were deduced to be candidates for additional LmrA targets.

For prediction of putative LmrA-binding sites, a web-based application, GRASP-DNA, was used (19; <http://www2.genomica.com/grasp-dna/>). The 36-bp sequence carrying the incomplete palindrome of the *lmr* promoter region (Fig. 1B) was used as a query sequence. GRASP-DNA automatically built a weight matrix based on the query, found sequences matching the matrix within the *B. subtilis* genome, and reported such sequences together with their adjacent genes. Among the reported sequences, those associated with at least one gene within the deduced LmrA target candidates were selected as putative LmrA-binding sites.

Northern hybridization. Northern blot analysis was performed as described previously (24). RNA samples were prepared in the same way that samples were prepared for the DNA microarray analysis. The probes were PCR products, corresponding to parts of *lmrA*, *lmrB*, *ypaG*, and *ypaH* (see Fig. 1A and 5A), that were amplified from *B. subtilis* strain 168 genomic DNA by using specific primer pairs and were labeled by using a BcaBest labeling kit (Takara Shuzo) with [α - 32 P]dCTP. The primer pairs employed were NlmrA1-NlmrA2 (for *lmrA*), NlmrB1-NlmrB2 (for *lmrB*), NyxaG1-NyxaG2 (for *ypaG*), and NyxaH1-NyxaH2 (for *ypaH*) (Table 1).

Assay for sensitivity to drugs and heavy metal ions. The sensitivity of *B. subtilis* strains to drugs and heavy metal ions was assayed as described previously (13). Cellular growth was monitored by measuring the optical density at 540 nm of cells grown in LB medium containing various concentrations of a drug or heavy metal ion. The concentration of the drug or ion that resulted in 50% growth inhibition was determined.

RESULTS

LmrA produced in *E. coli* binds specifically to the *lmr* promoter region. A DNA fragment containing the coding region of *B. subtilis* *lmrA* with its corresponding ribosome-binding site (Fig. 1A) was cloned into the multiple-cloning site of plasmid pUC18 to produce plasmid pLMRA, which placed *lmrA* under control of the *lac* promoter (22). When *E. coli* strain JM109 cells carrying pLMRA were grown in the presence of IPTG, we observed an extra protein band for the crude extract of the cells exhibiting the size (about 20 kDa) expected for LmrA (data not shown). The crude extract containing LmrA was used in gel retardation assays to examine the DNA binding of LmrA. As shown in Fig. 2B, the MK1-MK2 probe (Fig. 2A) carrying the entire *lmr* promoter region gave a distinct retarded band with the extract containing LmrA (Fig. 2B). The amount of this retarded band increased as the concentration of LmrA increased, while no such retarded band was observed with the most concentrated extract of JM109 cells carrying pUC18 that did not contain LmrA. Therefore, the retarded band most likely represented a specific LmrA-probe complex.

Identification of the LmrA-binding site. To identify the LmrA-binding site within the MK1-MK2 probe, a series of deleted probes (Fig. 2A) were prepared to examine the LmrA binding by the gel retardation assay. As shown in Fig. 2C, four probes (MK1-d1, MK1-d2, MK1-d3, and d5R-MK2) gave distinct retarded bands for LmrA-DNA complexes, while the other four probes (MK1-d4, MK1-d5, d3R-MK2, and d4R-MK2) did not. However, the MK1-d4 probe exhibited specific tailing in the presence of LmrA, implying that there was a weak interaction with LmrA (Fig. 2C). These results suggested that the region from position -36 to position 20 (position 1 is the transcription initiation nucleotide [11]) present in both of the shortest LmrA-interacting probes, MK1-d4 and d5R-MK2, could be required for the LmrA interaction (Fig. 2A). When the sequence of this region was examined, a 36-bp sequence comprising an incomplete palindrome sequence from position -18 to position 18 was found (Fig. 1). An internal deletion that eliminated the 36-bp region (del probe in Fig. 2A) abolished the LmrA binding completely (Fig. 2C). Furthermore, DNase I footprinting of LmrA in the *lmr* promoter region revealed that the regions from position -13 to position 11 of the non-coding strand (Fig. 3A, left gel) and from position -12 to position 12 of the coding strand (Fig. 3A, right gel) were protected from DNase I by the LmrA binding, and the pro-

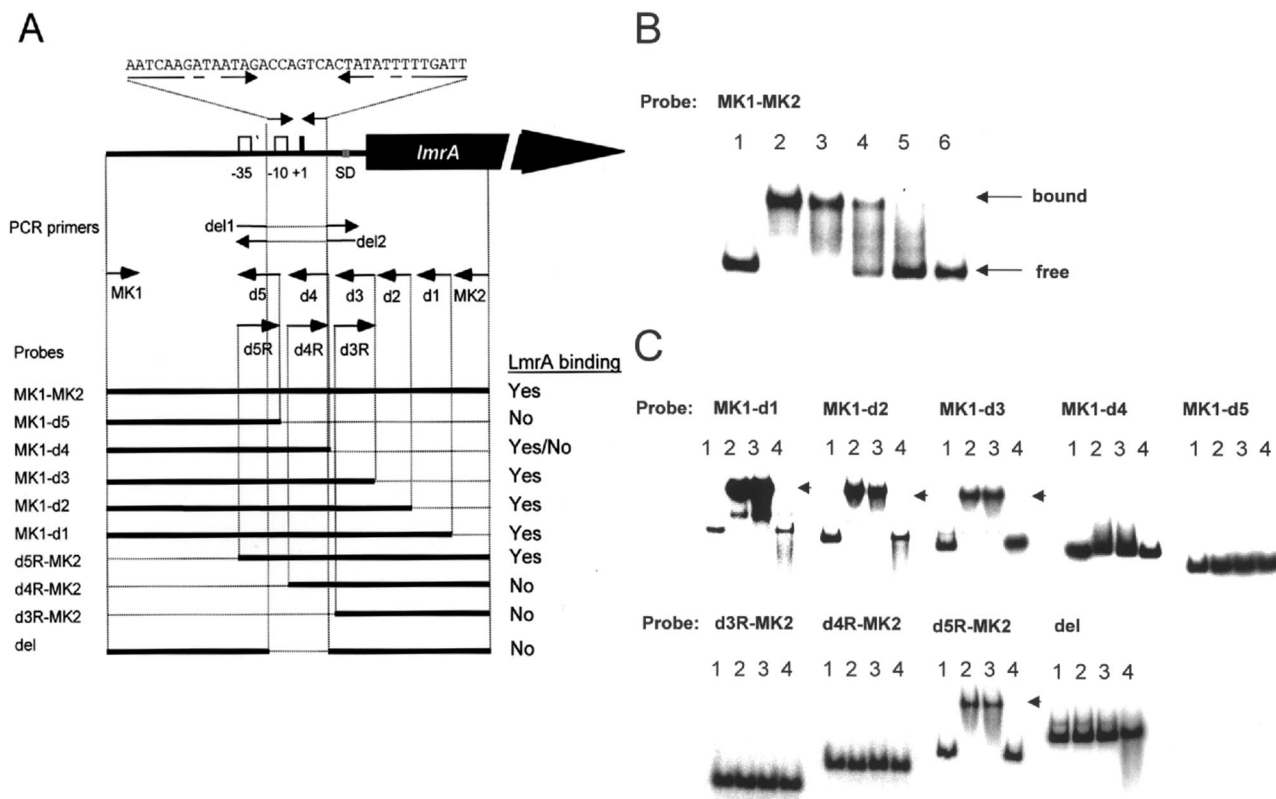


FIG. 2. Gel retardation analysis of LmrA binding to the *lmr* promoter region. (A) Probe settings. The positions and orientations of the PCR primers used for probe preparation are indicated schematically. The pair of horizontal arrows facing each other indicates the incomplete palindrome sequence. The probe designations are indicated on the left, and the thick horizontal lines and dotted lines indicate the stretches present and deleted in each of the probes, respectively. On the right, the results of gel retardation assay are summarized (Yes and No indicate LmrA binding and no LmrA binding, respectively). (B) Interaction between LmrA and the MK1-MK2 probe. The MK1-MK2 probe (0.02 pmol) was mixed with a protein extract of JM109 cells carrying plasmid pLMRA to obtain a reaction mixture (25 μ l) (lane 2, 7.5 μ g; lane 3, 3.8 μ g; lane 4, 1.9 μ g; lane 5, 0.9 μ g) or pUC18 (lane 6, 7.5 μ g) grown in the presence of 1 mM IPTG, and without the extract (lane 1). The positions of LmrA-probe complexes (bound) and free probe (free) are indicated on the right. (C) Deletion analysis. Gel retardation experiments were performed like the experiments described above. Each of the probes indicated was mixed with an extract of JM109 cells carrying pLMRA (lanes 2, 7.5 μ g; lanes 3, 3.8 μ g) or pUC18 (lanes 4, 7.5 μ g) or with no extract (lanes 1). The position of LmrA-probe complexes is indicated by arrows.

tected area was located in the middle of the region containing the palindrome sequence (Fig. 3B). Addition of an excess amount of lincomycin (10 mM) failed to abolish the LmrA binding (Fig. 3A, lane 6), suggesting that this drug does not act as an inducer that antagonizes the interaction between LmrA and DNA.

Mutations in the LmrA-binding site lowered LmrA affinity. Nonlabeled DNA fragments corresponding to the MK1-MK2 probe were amplified by PCR from DNA of strains 168, PLR1, and 1A221. The PLR1 and 1A221 fragments possessed mutations at the -1 position (A to T) and at both position -1 (A to C) and position 15 (G to T), respectively (Fig. 1). Specific competitors in the form of various amounts of each of the three fragments were added to gel retardation assay mixtures containing fixed amounts of the protein extract and the labeled MK1-MK2 probe. As shown in Fig. 4, the PCR fragment amplified from strain 168 DNA was able to compete with the labeled probe to decrease LmrA-probe complex formation as the amount was increased. Neither the fragment derived from PLR1 DNA nor the fragment derived from 1A221 DNA was found to be as competitive as the fragment derived from 168

DNA. The results suggested that the mutations of PLR1 and 1A221 could lower the LmrA-binding affinity.

***ysaGH* operon is in the LmrA regulon.** The mutated LmrA of strain PLR2 had completely lost repressor activity (13). In order to screen for additional LmrA candidate targets, DNA microarray analysis was carried out to compare the transcriptomes in strain PLR2 and 168 cells (data available at <http://www.genome.ad.jp/kegg/expression/>). On the other hand, a GRASP-DNA search (19) by using the 36-bp sequence carrying the incomplete palindrome sequence found in the *lmr* promoter region as a query was performed. This combined search revealed some additional candidates associated with a putative LmrA-binding site, such as *ysaG*. Gel retardation analyses to examine the interaction between LmrA and each of the putative binding sites revealed that LmrA strongly bound to a putative promoter region of the *ysaGH* operon (Fig. 5) but not to the other regions under the conditions which we used (data not shown). Northern analysis was performed to confirm that the levels of both the *ysaGH* and *lmrAB* transcripts were increased in PLR2; the *ysaG* and *ysaH* genes were cotranscribed as a 2.5-kb transcript (data not shown). These results

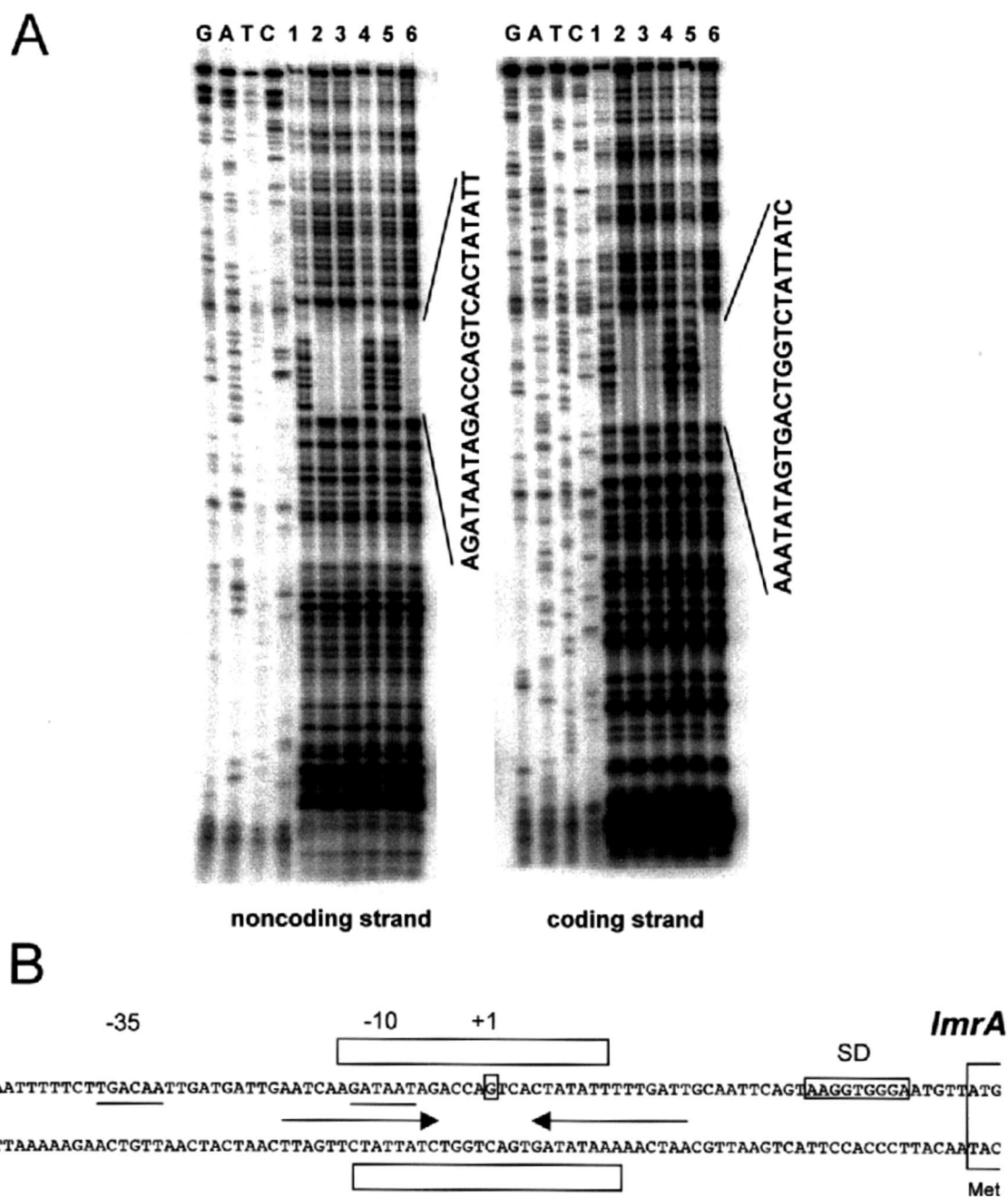


FIG. 3. DNase I footprinting of LmrA in the *lmr* promoter region. (A) DNase I footprinting of the noncoding (left gel) and coding (right gel) strands of the DNA of the *lmr* promoter region. The 5'-labeled probe (0.04 pmol) was combined in a reaction mixture (50 μ l) with protein extract (lanes 2, 4, and 6, 15.0 μ g; lanes 3 and 5, 7.5 μ g) prepared from JM109 cells carrying plasmid pLMRA (lanes 2, 3, and 6) or pUC18 (lanes 4 and 5) or with no extract (lane 1). The mixture loaded in lane 6 also contained 10 mM lincomycin. After DNase I digestion, samples were analyzed by sequencing 6% polyacrylamide gel electrophoresis. Areas protected by LmrA binding are indicated on the right of each gel, and the nucleotide sequences are shown. Lanes G, A, T, and C contained ladders created by dideoxy sequencing reactions with the corresponding 5'-labeled primers. (B) Summary of the DNase I footprint assay. The nucleotide sequences of the noncoding and coding strands of the *lmr* promoter region are shown. The -10 and -35 regions are underlined, and the transcription start site (position 1) and the Shine-Dalgarno sequence (SD) are enclosed in boxes. The facing horizontal arrows indicate the incomplete palindrome sequence. The protected areas found in the noncoding and coding strands are indicated by open boxes above and below the sequences, respectively.

indicated that *yxgH* was an additional LmrA target operon. DNase I footprint analysis of LmrA in the *yxgH* promoter region revealed two tandem LmrA-binding sites, *yxg* sites 1 and 2 (Fig. 6). *yxg* site 2 corresponded to the putative site predicted in the GRASP-DNA search, while the search failed

to predict site 1 because of its lower level of similarity to the query sequence (Fig. 7). Lincomycin did not interfere with the LmrA binding to either of these sites (Fig. 6A, lane 6). Putative -35 (TAGAAC) and -10 (GATAAT) sequences separated by a 17-bp spacer were found within the region covered by

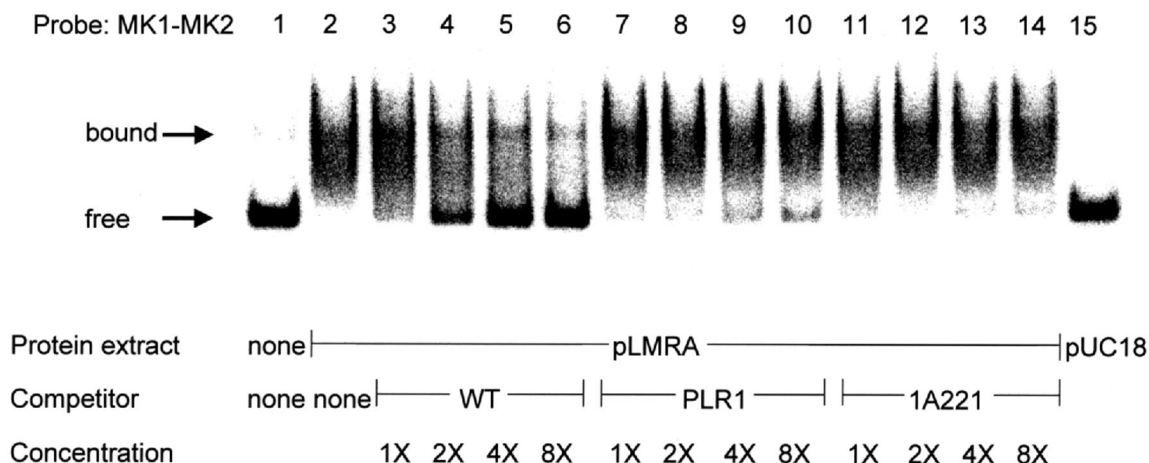


FIG. 4. Competition assay of LmrA binding between the wild-type DNA and mutated DNA of the *lmr* promoter region. The MK1-MK2 probe (0.02 pmol) was combined in the gel retardation assay reaction mixture (25 μ l) with an extract (3.8 μ g) of JM109 cells carrying plasmid pLMRA (lanes 2 to 14) or pUC18 (lane 15) or with no extract (lane 1). The specific competitors comprised nonlabeled PCR fragments corresponding to the MK1-MK2 probe amplified from DNA of strains 168 (WT) (lanes 3 to 6), PLR1 (lanes 7 to 10), and 1A221 (lanes 11 to 14), and the following amounts were added to the reaction mixture: 0.02 pmol (1 \times) (lanes 3, 7, and 11), 0.04 pmol (2 \times) (lanes 4, 8, and 12), 0.08 pmol (4 \times) (lanes 5, 9, and 13), and 0.16 pmol (8 \times) (lanes 6, 10, and 14). The positions of the LmrA-probe complex (bound) and free probe (free) are indicated by arrows.

LmrA (Fig. 6B). The two newly identified LmrA-binding sites of the *ypaGH* promoter region did not possess the palindrome sequence found in the binding site of the *lmr* promoter region. Instead, all three binding sites were found to share a conserved 18-bp consensus sequence, TAGACCRKTCWMTATAWT

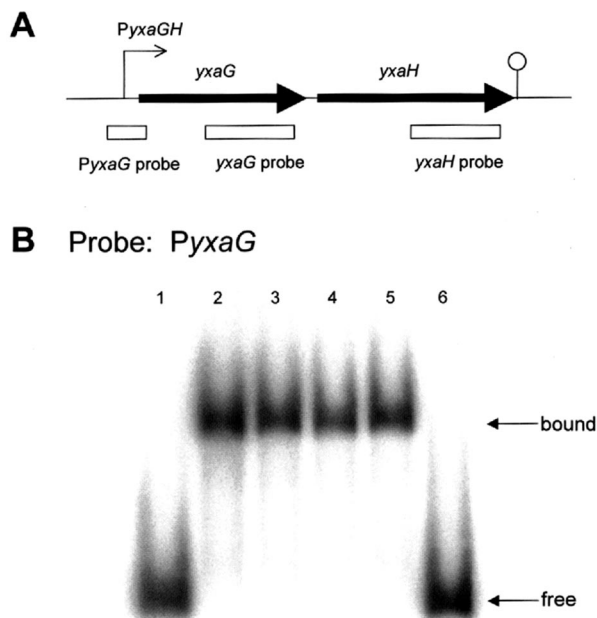


FIG. 5. Organization of the *ypaGH* operon and gel retardation analysis of LmrA binding to the putative promoter region. (A) Organization of the *ypaGH* operon. The regions corresponding to the PyxaG probe for gel retardation (see panel B) and the *ypaG* and *ypaH* probes for Northern analyses are indicated. (B) Gel retardation analysis of LmrA binding to the putative *ypaGH* promoter region. The conditions for the experiments and the lane assignments are the same as those described in the legend to Fig. 2B, except that the PyxaG probe was used.

(Fig. 7). This 18-bp sequence is located within and close to the middle of the 36-bp incomplete palindrome sequence of the *lmr* promoter region. In all three cases, the 18-bp sequence is located within the area protected from DNase I by LmrA binding. Conservation of this 18-bp sequence was not seen for the other putative binding sites predicted by the GRASP-DNA search, and LmrA did not interact with any of the probes carrying the other putative sites (data not shown). Moreover, another GRASP-DNA search involving all three conserved 18-bp sequences as queries failed to predict any more additional putative binding sites (data not shown). These results suggested that the conserved 18-bp region might play an important role in LmrA binding and that LmrA could regulate *lmrAB* and *ypaGH* exclusively within the genome.

Functional analysis of *lmrB* and *ypaGH*. Since LmrA is a repressor of *lmrAB* for multidrug resistance, we investigated the possibility that the second LmrA target, *ypaGH*, also contributed to drug resistance. Functional analysis of *lmrB*, *ypaG*, and *ypaH* was carried out in order to determine the extents of involvement of these genes in resistance to drugs and heavy metal ions. The growth rates of *B. subtilis* strains 168, PLR2, PLR3, PLR4, PLR5, PLR6, and PLR7 (Table 2) were compared in the presence of various chemicals (chloramphenicol, ampicillin, carbenicillin, erythromycin, lincomycin, puromycin, novobiocin, kanamycin, neomycin, spectinomycin, streptomycin, ofloxacin, norfloxacin, levofloxacin, tosufloxacin, mitomycin C, daunorubicin, doxorubicin, rifampin, verapamil, bicyclomycin, acriflavine, acridine orange, crystal violet, ethidium bromide, rhodamine 6G, proflavine, pyronin Y, Cd²⁺, Hg²⁺, Zn²⁺, and Co²⁺). Mutant PLR2 (which expresses both *lmrB* and *ypaGH*) was found to exhibit multidrug resistance, as reported previously (13) (data for lincomycin and puromycin are shown in Table 2), while PLR3 (which possesses the *lmrB* inactivation locus [*lmrB::cat*] but expresses *ypaGH*) was found to have almost completely lost the resistance phenotype. These results suggest that *ypaGH* might not contribute to multidrug resistance.

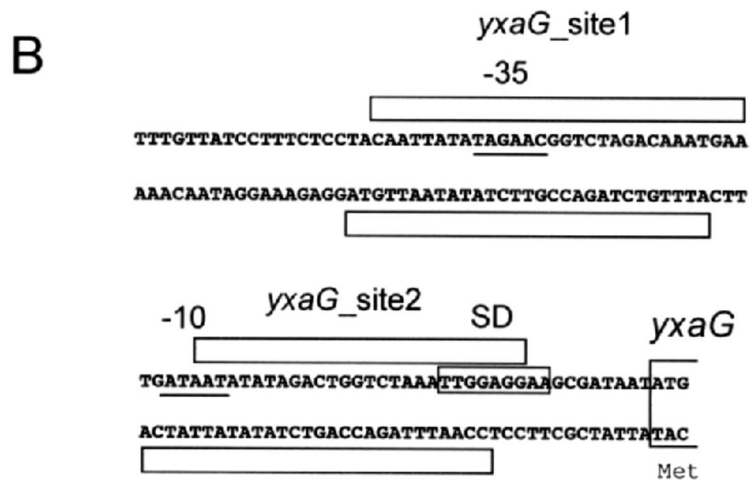
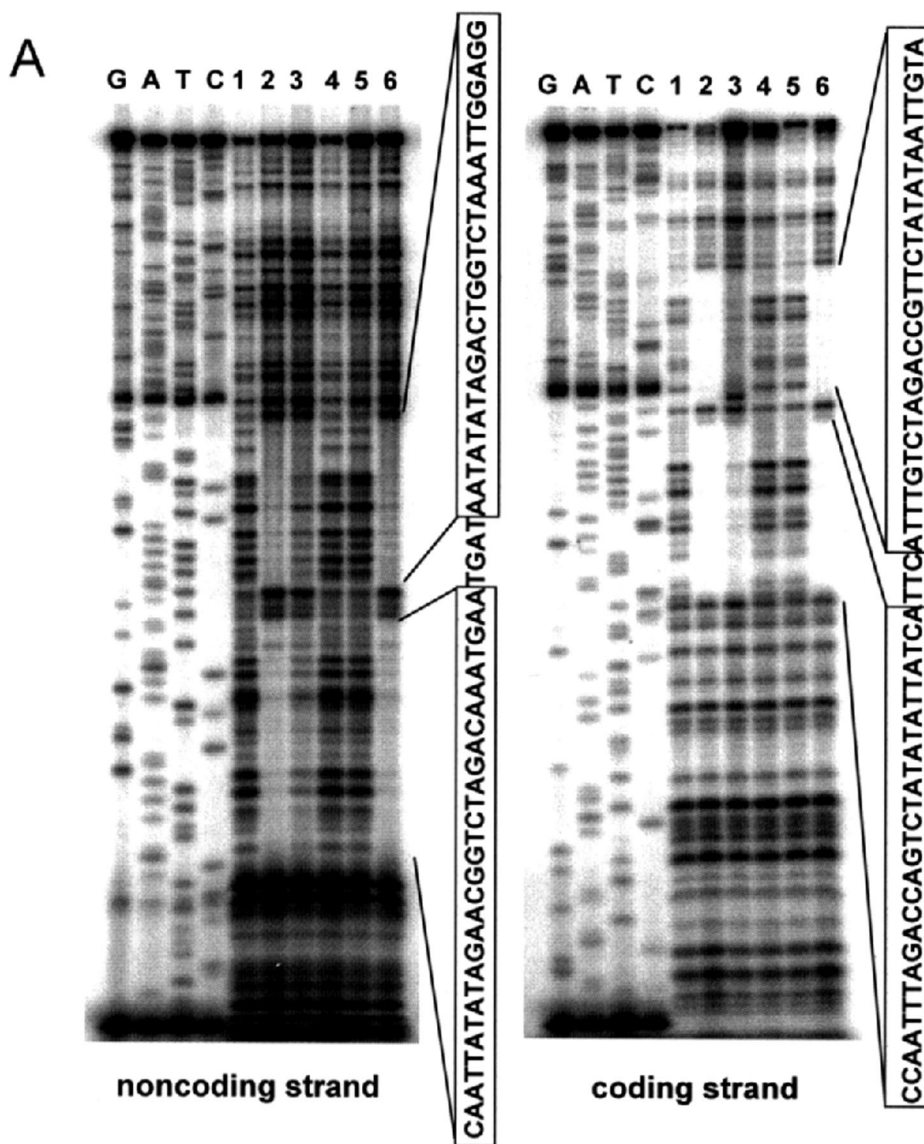


FIG. 6. DNase I footprinting of LmrA in the putative *yxaGH* promoter region. (A) DNase I footprinting in the *yxaGH* promoter region. The conditions for the experiments and the lane assignments are the same as those described in the legend to Fig. 3A, except for the probes (see text).

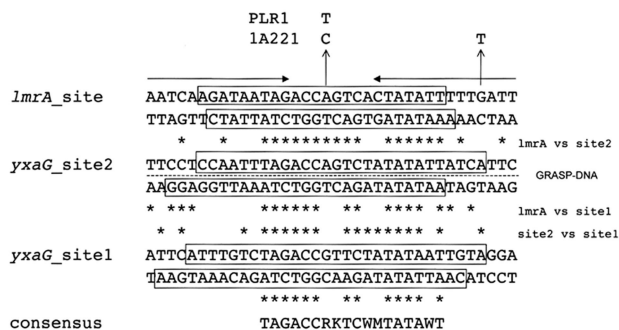


FIG. 7. Alignment of the nucleotide sequences of the three LmrA-binding sites. The 36-bp nucleotide sequences of both strands of the three LmrA-binding sites (*lmrA* site, *yxaG* site 1, and *yxaG* site 2) are aligned. To optimize the alignment, the sequences of the *lmrA* site are oriented so that transcription occurs from left to right, while those of *yxaG* site 1 and *yxaG* site 2 are in the opposite orientation. The nucleotide positions conserved in two of the three sites are indicated by asterisks on the lines labeled *lmrA* vs site2 (between the *lmrA* site and *yxaG* site 2), *lmrA* vs site1 (between the *lmrA* site and *yxaG* site 1), and site2 vs site1 (between *yxaG* site 2 and *yxaG* site 1). Beneath the alignment, the 18-bp consensus sequence for the three sites is indicated by asterisks that indicate the positions conserved in the three sites. The protected areas found in the DNase I footprinting experiments are enclosed in boxes. The pair of horizontal arrows facing each other above the sequence of the *lmrA* site indicates the incomplete palindrome sequence. The dotted line beneath the upper sequence of *yxaG* site 2 indicates that this sequence was predicted to be a putative LmrA-binding site during the GRASP-DNA search. The vertical arrows indicate the mutation points found in strains PLR1 and 1A221.

In addition, the pMUTIN2 integration disrupting either *yxaG* or *yxaH* (PLR4 and PLR6 in a PLR2 background and PLR5 and PLR7 in a PLR3 background) did not cause a significant change in the sensitivity to puromycin (Table 2) or the other chemicals tested (data not shown) except for erythromycin, lincomycin, and Hg²⁺. The pMUTIN2-borne *erm* gene (20) conferred resistance to macrolides such as erythromycin and lincomycin; thus, the 50% inhibitory concentrations of lincomycin for the pMUTIN2 integrants could not be determined (Table 2) (at least 70% of the cells were viable at the highest concentration tested, 2 mg/ml). Unexpectedly, the Hg²⁺ sensitivity of the cells was elevated after disruption of either *yxaG* or *yxaH* only in a PLR2 background (PLR4 and PLR6), in which the *lmrA* mutation allowed *lmrB* expression (Table 2).

DISCUSSION

In this study the LmrA-binding site for repression of the *lmr* promoter was identified by gel retardation and DNase I footprint analyses (Fig. 2 and 3). DNA microarray and gel retardation analyses revealed that *yxaGH* acted as an additional LmrA target (Fig. 5). Two tandem LmrA-binding sites were identified within the putative *yxaGH* promoter region in the DNase I footprinting experiments (Fig. 6). All three LmrA-binding sites were found to possess an 18-bp consensus se-

quence, TAGACCRKTCWMTATAWT (Fig. 7). However, the results of gel retardation competition assays (Fig. 4) suggested that strain 1A221 DNA carrying the double mutations at positions -1 (A to C) and 15 (G to T) could have slightly lower affinity for LmrA than the PLR1 DNA with the single mutation at position -1 (A to T) has (Fig. 4), and thus the mutation at position 15 outside the 18-bp sequence (Fig. 7) might also be involved in LmrA binding. In addition, the MK1-d4 probe, including the 18-bp sequence, failed to yield a distinct LmrA-probe complex, as judged by the gel retardation experiment (Fig. 2). Nevertheless, the MK1-d4 probe band exhibited tailing in the presence of LmrA (Fig. 2), implying that an incomplete interaction may have occurred. Therefore, it is likely that tight binding of LmrA to the *lmr* promoter region requires not only the 18-bp sequence but also some extended region. A further point for consideration is that the tandem LmrA-binding sites in the *yxaGH* promoter region were found to be located close to each other (Fig. 6). It is possible that they might enhance the affinity of LmrA binding in a cooperative fashion. More precise studies are needed to clarify these findings.

None of the broad range of chemicals which we tested (including lincomycin) interfered with the interaction between LmrA and the three binding sites identified above (Fig. 3 and 6) (data not shown). Therefore, at present, no factors other than mutations have been found to induce the LmrA-repressed genes. However, it is very possible that an unidentified inducer could be responsible for the inactivation of the repressor function of LmrA, allowing expression of LmrA targets. Furthermore, it is possible that LmrA-repressed genes might be induced only by mutations in the binding sites and/or the *lmrA* coding region, as found for spontaneous drug-resistant mutants PLR1, PLR2, 1A221, etc. (11, 13). A similar situation has been reported for the *Streptomyces coelicolor* A3(2) *pqrAB* operon regarding paraquat resistance (6). PqrA is a TetR family repressor of its own operon, and PqrB is a putative efflux transporter of paraquat. Mutations in *pqrA* fully induced *pqrAB* transcription, while paraquat could only slightly induce transcription, but the precise mechanisms underlying the regulation were not clarified.

Our results provided no evidence for involvement of *yxaGH* in multidrug resistance. However, the Hg²⁺ sensitivity of the cells was elevated after disruption of either *yxaG* or *yxaH* only in a PLR2 background (PLR4 and PLR6), in which the *lmrA* mutation allowed *lmrB* expression (Table 2). At present, we are unable to properly explain why *yxaGH* is involved in Hg²⁺ resistance only with *lmrB* expression. Very recently, we noticed that YxaG is an iron-containing quercetin 2,3-dioxygenase, which converts the flavonol quercetin into 2-protocatechuoyl-phloroglucinol carboxylic acid and carbon monoxide (4, 5). Quercetin is one of the most abundant natural flavonoids inhibiting bacterial DNA gyrase that induces DNA cleavage (17), and thus YxaG might function in the detoxification of this compound. Our results clearly indicated that *yxaGH* forms an

Protected areas are enclosed in boxes in the nucleotide sequence on the right of each gel. (B) Summary of the DNase I footprint assaying. The nucleotide sequences of both strands of the *yxaGH* promoter region are shown. The putative -10 and -35 regions are underlined, and the putative Shine-Dalgarno sequence (SD) is enclosed in a box. The protected areas of *yxaG* site 1 and *yxaG* site 2 are indicated by open boxes.

TABLE 2. Growth inhibition of *B. subtilis* strains in the presence of lincomycin, puromycin, and Hg²⁺

Strain	Relevant genotype	IC ₅₀ ^a		
		Lincomycin (μg/ml)	Puromycin (μg/ml)	Hg ²⁺ (μM)
168	Wild type	8.5 ± 2.9	14.4 ± 5.1	7.2 ± 1.4
PLR2	<i>ImrA</i> (Q52P stop to S)	106.6 ± 25.3	474.6 ± 57.7	6.6 ± 1.3
PLR3	<i>ImrA</i> (Q52P stop to S) <i>ImrB::cat</i>	3.8 ± 0.5	7.3 ± 1.4	8.3 ± 2.1
PLR4	<i>ImrA</i> (Q52P stop to S) <i>yxaG::pMUTIN2</i>	ND ^b	464.6 ± 23.8	3.4 ± 1.1
PLR5	<i>ImrA</i> (Q52P stop to S) <i>ImrB::cat yxaG::pMUTIN2</i>	ND	9.2 ± 0.8	9.5 ± 0.7
PLR6	<i>ImrA</i> (Q52P stop to S) <i>yxaH::pMUTIN2</i>	ND	468.4 ± 18.4	3.4 ± 1.1
PLR7	<i>ImrA</i> (Q52P stop to S) <i>ImrB::cat yxaH::pMUTIN2</i>	ND	9.5 ± 0.9	10.2 ± 0.3

^a IC₅₀, concentration of the drug or ion that gave 50% growth inhibition. The values are mean ± standard deviations for at least four independent measurements.

^b ND, not determined under the assay conditions employed, due to the presence of the pMUTIN2-borne *erm* gene (20), which is known to confer resistance to lincomycin, as well as erythromycin, on the cells.

operon, and YxaH, a putative membrane protein with nine transmembrane segments (BSORF website), might act in a cooperative manner with YxaG as a drug exporter. In future investigations we will focus on these possibilities.

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