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BIODEGRADATION

McbG, a LysR Family Transcriptional Regulator, Activates the *mcbBCDEF* Gene Cluster Involved in the Upstream Pathway of Carbaryl Degradation in *Pseudomonas* sp. Strain XWY-1

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ABSTRACT Although enzyme-encoding genes involved in the degradation of carbaryl have been reported in *Pseudomonas* sp. strain XWY-1, no regulator has been identified yet. In the *mcbABCDEF* cluster responsible for the upstream pathway of carbaryl degradation (from carbaryl to salicylate), the *mcbA* gene is constitutively expressed, while *mcbBCDEF* is induced by 1-naphthol, the hydrolysis product of carbaryl by McbA. In this study, we identified McbG, a transcriptional activator of the *mcbBCDEF* cluster. McbG is a 315-amino-acid protein with a molecular mass of 35.7 kDa. It belongs to the LysR family of transcriptional regulators and shows 28.48% identity to the pentachlorophenol (PCP) degradation transcriptional activation protein PcpR from *Sphingobium chlorophenolicum* ATCC 39723. Gene disruption and complementation studies reveal that *mcbG* is essential for transcription of the *mcbBCDEF* cluster in response to 1-naphthol in strain XWY-1. The results of the electrophoretic mobility shift assay (EMSA) and DNase I footprinting show that McbG binds to the 25-bp motif in the *mcbBCDEF* promoter area. The palindromic sequence TATCGATA within the motif is essential for McbG binding. The binding site is located between the –10 box and the transcription start site. In addition, McbG can repress its own transcription. The EMSA results show that a 25-bp motif in the *mcbG* promoter area plays an important role in McbG binding to the promoter of *mcbG*. This study reveals the regulatory mechanism for the upstream pathway of carbaryl degradation in strain XWY-1. The identification of McbG increases the variety of regulatory models within the LysR family of transcriptional regulators.

IMPORTANCE *Pseudomonas* sp. strain XWY-1 is a carbaryl-degrading strain that utilizes carbaryl as the sole carbon and energy source for growth. The functional genes involved in the degradation of carbaryl have already been reported. However, the regulatory mechanism has not been investigated yet. Previous studies demonstrated that the *mcbA* gene, responsible for hydrolysis of carbaryl to 1-naphthol, is constitutively expressed in strain XWY-1. In this study, we identified a LysR-type transcriptional regulator, McbG, which activates the *mcbBCDEF* gene cluster responsible for the degradation of 1-naphthol to salicylate and represses its own transcription. The DNA binding site of McbG in the *mcbBCDEF* promoter area contains a palindromic sequence, which affects the binding of McbG to DNA. These findings enhance our understanding of the mechanism of microbial degradation of carbaryl.

KEYWORDS 1-naphthol, McbG, *Pseudomonas* sp. XWY-1

Carbaryl (1-naphthyl-*N*-methylcarbamate) is a carbamate insecticide widely used in agricultural and forestry pest control (1). However, carbaryl residues are considered an environmental pollutant because carbaryl inhibits the activity of acetylcholinesterase

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and poses a potential threat to humans and other nontarget organisms; therefore, it has attracted increasing attention (2). Bioremediation has received increasing attention as a reliable and environmentally friendly approach to clean up polluted environments (3), and research on the mechanism of microbial degradation of carbaryl will help in the bioremediation of carbaryl polluted environments. To date, several carbaryl-degrading strains have been reported from the genera *Pseudomonas* (4–7), *Sphingobium* (8), *Novosphingobium* (9, 10), *Rhizobium* (11, 12), *Pseudaminobacter* (13), *Rhodococcus* (14), *Achromobacter* (15), and *Arthrobacter* (16). Among these, the mechanisms of carbaryl degradation in *Pseudomonas* sp. strain C5pp and *Pseudomonas* sp. strain XWY-1 have been extensively investigated (17, 18).

The complete degradation pathway of carbaryl has been elucidated in strain C5pp, including the upstream pathway from carbaryl to salicylate, the midstream pathway from salicylate to gentisate, and the downstream pathway from gentisate to pyruvate and fumarate (18). The genome of this strain has been sequenced, and the enzyme-encoding genes involved in the degradation have also been identified (18, 19). However, no regulator of this pathway has yet been identified. Strain XWY-1 was isolated in our lab and utilized carbaryl and its metabolite 1-naphthol as sole carbon sources for growth. Moreover, strain XWY-1 degraded carbaryl through the same pathway as strain C5pp. Comparison of the genomes of strains XWY-1 and C5pp showed that it also harbors the same *mcbABCDEFG*, *mcbHIJKLM*, and *mcbNOPQ* clusters as strain C5pp, which encodes the entire degradation pathway of carbaryl. Like strain C5pp, no regulatory genes have yet been identified for the carbaryl degradation pathway in strain XWY-1 (Fig. 1) (17).

In the *mcbABCDEF* cluster responsible for the upstream pathway of carbaryl degradation, the *mcbA* gene responsible for hydrolysis of carbaryl to 1-naphthol is constitutively expressed in strain XWY-1, while the *mcbBCDEF* cluster, responsible for the degradation of 1-naphthol to salicylate, is induced by 1-naphthol (20). In the present study, a LysR family transcriptional regulator, McbG, was identified as the transcriptional activator of the *mcbBCDEF* cluster in response to 1-naphthol in strain XWY-1 by using DNA alignment. The regulatory mechanism, including the transcription start site (TSS), the binding site, the core binding sequence, and the effect of substrate on its binding were investigated. In addition, the regulatory mechanism of *mcbG* itself by 1-naphthol was also investigated. The identification of McbG deepens our understanding of the regulatory mechanisms of carbaryl degradation.

RESULTS

Determination of the TSS of the *mcbBCDEF* cluster. The promoter of the *mcbBCDEF* cluster was predicted by the Berkeley Drosophila Genome Project (BDGP) Neural Network Promoter Prediction online program (http://www.fruitfly.org/seq_tools/promoter.html) with a score of >0.8 in the region upstream of the *mcbF* gene. The T was the 69th base upstream of the translational start codon of *mcbF*. The −10 box TGGTATTAT and the −35 box TTTTCA were predicted based on the identified TSS (Fig. 2).

All of the six fragments (F1 to F6) within the *mcbBCDEF* cluster were amplified using cDNA derived from strain XWY-1 induced by 1-naphthol as the template (Fig. 3A and B), indicating that the *mcbB*, *mcbC*, *mcbD*, *mcbE*, and *mcbF* genes were in one operon and transcribed in a single unit.

McbG is a transcriptional activator of the *mcbBCDEF* cluster. McbG was discovered upon alignment of the genomes of strain XWY-1 and strain C5pp. There was 100% identity between McbG of strains XWY-1 and C5pp. However, among the previously identified regulatory proteins (the UniProt Knowledge Base/Swiss-Prot databases), McbG shared the highest similarity (28.48%) only with the LysR family transcriptional regulator PcpR (GenBank accession number P52679.2) from the pentachlorophenol (PCP)-degrading strain *Sphingobium chlorophenolicum* ATCC 39723 (see Fig. S1 in the supplemental material). McbG contains 315 amino acids with a predicted molecular mass of 35.7 kDa. The N-

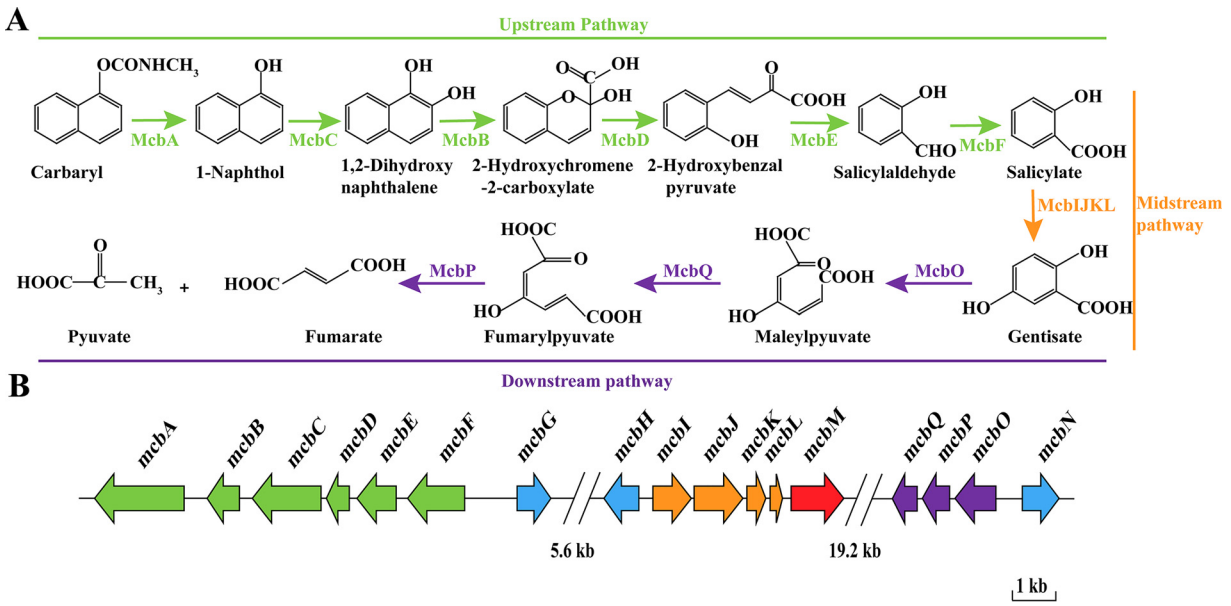

FIG 1 The carbaryl metabolism pathway and the involved genes in strain XWY-1. (A) The carbaryl degradation pathway. The green line indicates the upstream pathway, the orange line indicates the midstream pathway, and the purple line indicates the downstream pathway. (B) The involved carbaryl-degrading gene clusters. The green arrow indicates the gene cluster for upstream pathway, the orange arrow indicates the gene cluster for midstream pathway, the purple arrow indicates the gene cluster for downstream metabolism, the blue arrow indicates the putative regulatory protein, and the red arrow indicates the putative transporter. McbG shared the highest similarity (28.48%) with the LysR family transcriptional regulator PcpR (GenBank accession number P52679.2), McbH shared the highest similarity (68.67%) with the HTH-type transcriptional activator NahR (P10183.2), McbN shared the highest similarity (36.01%) with the HTH-type transcriptional regulator GbpR (P52661.1), and McbM shared the highest similarity (42.39%) with the 3-hydroxybenzoate transporter MhbT (Q5EXK5.1).

terminal amino acids 18 to 77 form a helix-turn-helix (HTH_XRE superfamily) domain, which is characteristic of transcriptional regulators.

The *mcbG* gene of strain XWY-1 was deleted to generate a knockout strain, MT. A complementation strain, MTC, was generated by transforming MT with plasmid pB*mcbG*. Cells of strains XWY-1, MT, and MTC were incubated with or without 1-naphthol. The transcription levels of the *mcbB*, *mcbC*, *mcbD*, *mcbE*, and *mcbF* genes were evaluated. As shown in Fig. 4, when supplemented with 1-naphthol, the transcription levels of *mcbB*, *mcbC*, *mcbD*, *mcbE*, and *mcbF* in strain XWY-1 were 171-, 178-, 169-, 134-, and 143-fold higher, respectively, than in the absence of 1-naphthol. In the knockout strain MT, the transcription levels of these genes were similar in the presence or absence of 1-naphthol. In the *mcbG*-complemented strain MTC, the transcription levels were similar to the wild-type strain XWY-1 (Fig. 4).

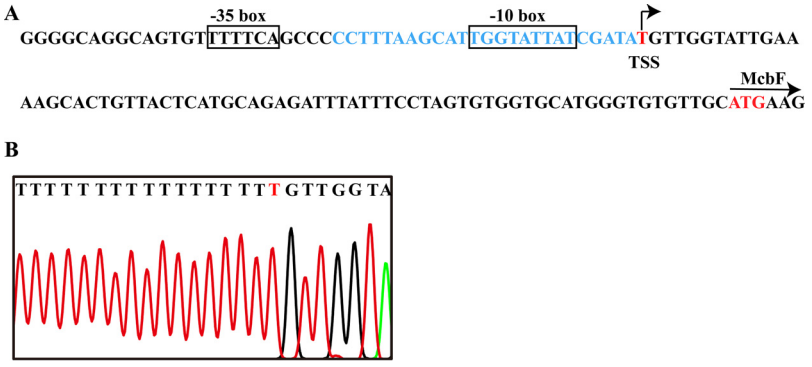

FIG 2 (A) DNA elements in the promoter of *mcbBCDEF* cluster. The −35 and −10 boxes are shown in boxes, and the TSS is shown by an arrow. The McbG-binding site is indicated by the blue sequence. (B) Chromatograms display the partial sequences of the 5′ RACE products. The red letter T indicates the TSS.

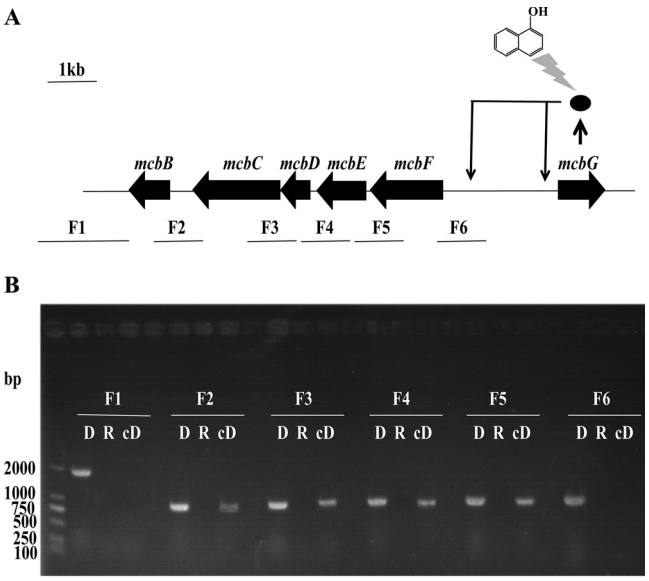

FIG 3 (A) Schematic diagram of the transcriptional regulation of McbG. The DNA fragments that are located at same positions in the genome are shown. The ellipse represents the protein McbG, which activates the transcription of the *mcbBCDEF* cluster and releases the self-repression of McbG in the presence of 1-naphthol (displayed as a solid line). The scale bar represents 1 kb. The amplification fragments for transcriptional unit evaluation are shown under the *mcbBCDEF* cluster as lines. (B) PCR amplification of the *mcbBCDEF* cluster using DNA (D), total RNA (R), and cDNA (cD) as the templates. The amplified products were detected by electrophoresis.

Cells of the strains XWY-1, MT, and MTC were incubated in mineral salts medium (MSM) supplemented with 0.1 mM 1-naphthol as the sole carbon source. As shown in Fig. S2, strain MT lost its ability to degrade 1-naphthol, which was regained in strain MTC carrying plasmid pB*mcbG* to levels similar to that of strain XWY-1. These results demonstrate that *mcbG* is essential for degradation of 1-naphthol in strain XWY-1.

McbG binds to *mcbBCDEF* promoter DNA. The *mcbG* was overexpressed in *Escherichia coli* BL21(DE3), and McbG was purified. Purified McbG appeared as a single

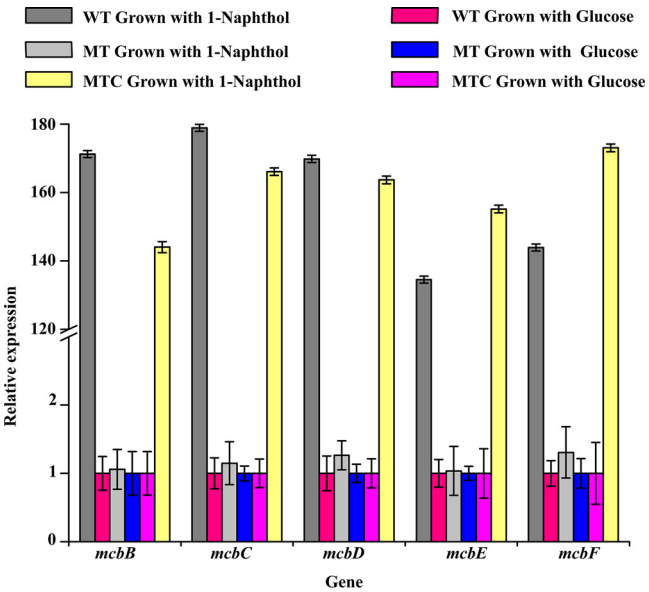

FIG 4 Transcriptional analysis of *mcbB*, *mcbC*, *mcbD*, *mcbE*, and *mcbF* in strains XWY-1 (WT), the *mcbG* knockout mutant (MT), and the *mcbG*-complemented strain (MTC) in the presence of 0.1 mM glucose or 0.1 mM 1-naphthol. The transcriptional level of the 16S rRNA gene was used as an internal standard, and the data in each column were calculated with the $2^{-\Delta\Delta CT}$ threshold cycle (C_T) method using three replicates.

band on SDS-PAGE, with a molecular mass of 35.7 kDa, which is in agreement with its theoretical molecular mass (Fig. S3). McbG was subjected to an electrophoretic mobility shift assay (EMSA) to test its binding capacity toward *mcbBCDEF* promoter DNA. When 50 nM McbG was added, a DNA-protein complex was observed (Fig. 5A). No DNA-protein complex was detected for the nonspecific control DNA (partial sequence of *mcbC*). When 1-naphthol was incubated with the promoter of *mcbBCDEF* and McbG, the binding capacity of McbG to the promoter was improved, especially at a lower concentration of 30 nM (Fig. 5A). These results indicate that McbG can bind to the *mcbBCDEF* promoter DNA, and 1-naphthol can enhance this binding.

1-Naphthol as effector to activate *mcbBCDEF* cluster transcription. To determine the effector of McbG, the *mcbC* gene, responsible for converting 1-naphthol to 1,2-dihydroxynaphthalene (Fig. 1), was knocked out to generate strain XWY-1 Δ*mcbC*. A reporter plasmid, pME6522-P_{mcb} (the fragment upstream of TSS of *mcbBCDEF* was fused with *lacZ* in the promoter probe plasmid pME6522 [21] to generate to pME6522-P_{mcb}), was then introduced into strain XWY-1 Δ*mcbC*. The strain was then cultured in GM (MSM with glucose as the sole carbon source) with or without 1-naphthol. Very low β-galactosidase activity ($<$65 Miller units) was detected in strain XWY-1 Δ*mcbC* grown in the absence 1-naphthol, while approximately 1,700 Miller units of activity was observed in XWY-1 Δ*mcbC* grown in the presence of 1-naphthol (Fig. 5B). These results indicate that 1-naphthol is an effector of the *mcbBCDEF* cluster and not its subsequent metabolites.

Binding site of McbG to the *mcbBCDEF* promoter. A DNase I footprinting assay was performed to identify the McbG-binding site in the promoter region of *mcbBCDEF*. It was found that McbG protected the 25-bp motif CCTTTAAGCATTGGTATTATCGATA (Fig. 6A), spanning base pairs −1 to −25 in the *mcbBCDEF* promoter (Fig. 2A), and was located between the −10 box and the transcription start site (Fig. 2A). Upon deletion of the 25-bp motif, EMSA results revealed that McbG could no longer bind to the DNA probe (Fig. 6B). The 25-bp motif harbors a palindromic sequence, 5′-TATCGATA-3′. To determine whether this palindromic sequence is essential for McbG binding, it was mutated to 5′-TTTAACCC-3′. EMSA analysis showed that the mutated DNA probe no longer bound to McbG (Fig. 6C). Additionally, to determine the role of other 17-bp sequences in binding, the 25-bp motif was mutated to AGGTAAGGTAAGGTAATTATCGATA (mutated sequences underlined). When this was used as a probe, a weakening of cohesion was observed (Fig. 6C). These results indicate that the palindromic sequence plays an important role in McbG sequence recognition.

1-Naphthol relieves the self-repression of McbG. To explore whether McbG regulated the transcription of *mcbG* itself, the promoter of the *mcbG* was analyzed (Fig. 7A). The cells of strain XWY-1 were incubated with or without 1-naphthol. The transcription levels of *mcbG* genes were evaluated. The reverse transcription-quantitative PCR (qRT-PCR) results showed that the transcription level of *mcbG* increased significantly (4 times) at 1.5 h and then dropped to an extremely low level (Fig. S4). The results of the EMSA revealed that McbG bound to its own promoter. When 1-naphthol was added as the effector, the binding ability of McbG with the promoter was weakened, indicating that McbG might repress its own expression, while 1-naphthol relieved the repression (Fig. 7B). To determine the McbG binding site, the different, partially overlapping subfragments F1, F2, and F3, were used for the EMSA (Fig. 7C). The F1 subfragment was completely shifted after incubation with McbG (200 nM), whereas subfragments F2 and F3 were not (Fig. 7D). To precisely define the binding region, the subfragments of a and b from F1 were used for the EMSA (Fig. 7E). Only subfragment a, which contained a 25-bp motif (5′-CCTCCAACAATATTGATATATTTTA-3′) was shifted in the presence of McbG (Fig. 7E). These results indicate that this motif plays a key role in promoter binding of McbG.

DISCUSSION

McbG, a LysR family transcriptional activator, was identified in this study. The knockout of *mcbG* caused a loss of 1-naphthol degradation ability in strain XWY-1

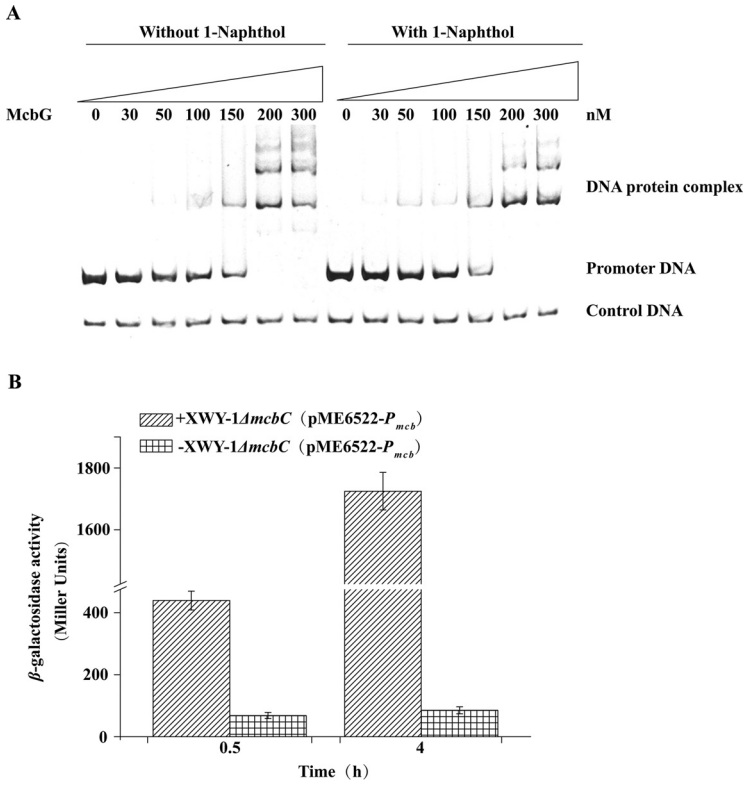

FIG 5 (A) Electrophoretic mobility shift assays on the binding of McbG to the *mcbBCDEF* cluster promoter. Each lane contains 20 ng of DNA probe. The first 7 lanes show samples incubated without 1-naphthol, and the next 7 lanes show samples incubated with 0.05 mM 1-naphthol. The concentrations of McbG, increasing from left to right, are shown above the lanes. The control DNA was a 200-bp fragment that was amplified from the *mcbC* gene. (B) *In vivo* inducer identification. A β-galactosidase assay was performed with XWY-1 Δ*mcbC* (pME6522-P_{mcb}) carrying the P_{mcb}-*lacZ* transcriptional fusion, grown in the presence (+XWY-1 Δ*mcbC* [pME6522-P_{mcb}]) or absence (−XWY-1 Δ*mcbC* [pME6522-P_{mcb}]) of 0.1 mM 1-naphthol. β-Galactosidase activity was measured as described in Materials and Methods. Each value is the mean ± standard deviation (SD) of at least three cultures.

(Fig. S2). The qRT-PCR results also revealed no significant differences in the transcription of each gene in the *mcbBCDEF* cluster in the cells of the knockout strain MT in cultures with and without induction of 1-naphthol (Fig. 4). These results indicate that McbG is a regulatory protein that is involved in the degradation of 1-naphthol and activates the transcription of *mcbBCDEF*. The results of the EMSA showed that McbG binds to the promoter of *mcbBCDEF*. Furthermore, upon addition of 1-naphthol, McbG binds to the promoter DNA even at low concentrations down to 30 nM (Fig. 5A). Effector assays using plasmid pME6522-P_{mcb} in the mutant strain XWY-1 Δ*mcbC* also showed that the activity of β-galactosidase in the presence of 1-naphthol was significantly higher than in its absence (Fig. 5B), indicating that the effector of McbG is 1-naphthol and not any of its follow-up products.

McbG was found to share similarity with LysR transcription regulators (Fig. S1). Interestingly, LysR-type regulators, including McbG, are involved in the degradation of pollutants in bacteria (22). Examples of this family include CatR, involved in the degradation of catechol in *Pseudomonas putida* (23), PnpR and PnpM of *Pseudomonas* sp. strain WBC-3, involved in the degradation of *para*-nitrophenol (24, 25), PcpR, involved in the degradation of pentachlorophenol in *Sphingobium chlorophenolicum* ATCC 39723 (26), and NagR, involved in the degradation of gentisate in *Ralstonia* sp. strain U2 (27). In addition, the binding of McbG to *mcbBCDEF* promoter DNA was effector independent (Fig. 5A). This phenomenon is characteristic of LysR-type transcriptional regulators (28).

The binding sites of LysR family transcriptional regulators were initially reported in *Rhizobium* spp., and a specific palindromic sequence, ATC-N_9-GAT, was identified. This

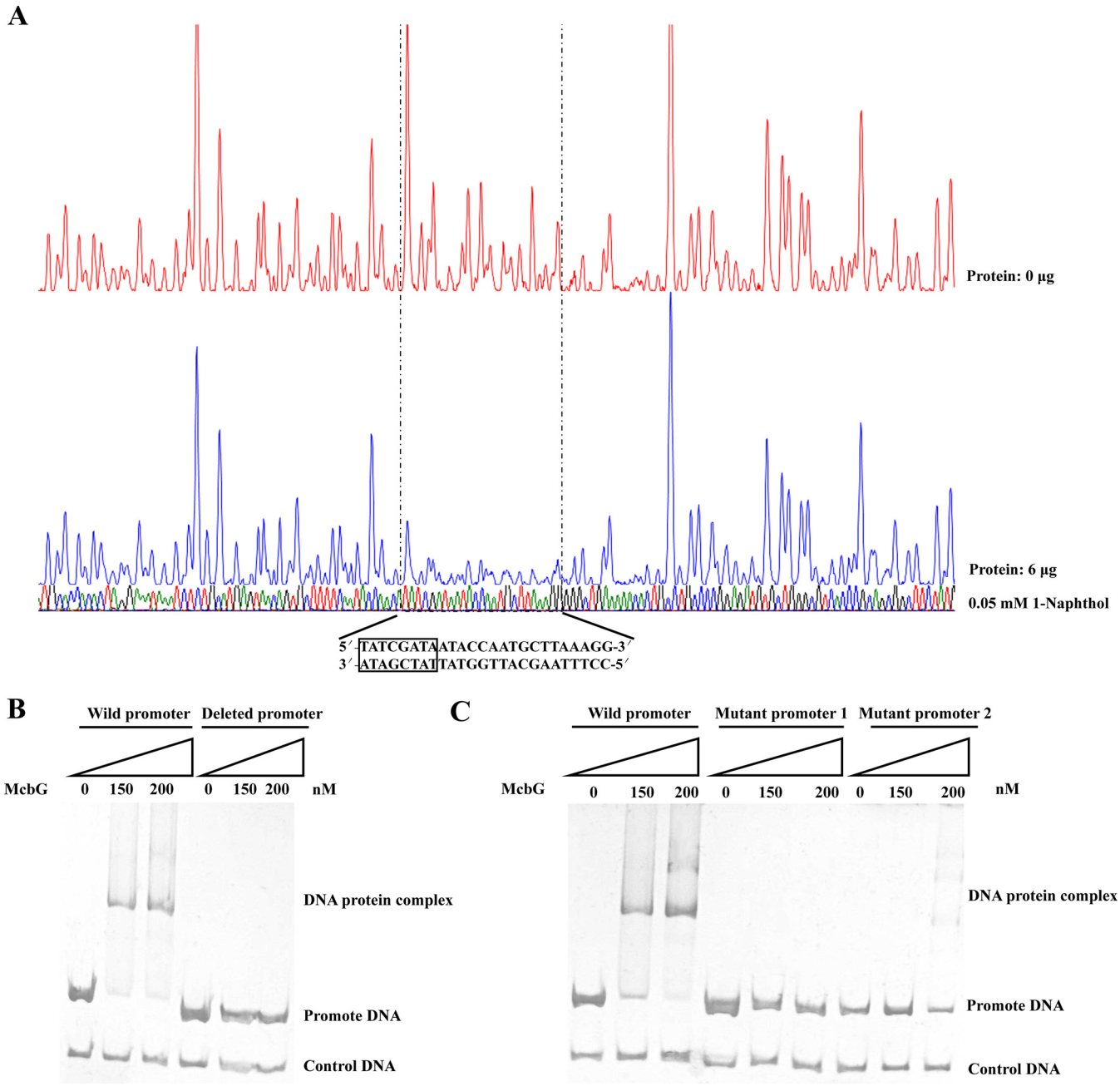

FIG 6 (A) DNase I footprinting analysis of the McbG-binding site in the *mcbBCDEF* promoter. A total of 400 ng of 6-carboxyfluorescein-labeled DNA probe was incubated without McbG (red line) or with 6 μg McbG (blue line) in the presence of 0.05 mM 1-naphthol. The McbG-protected region is shown in the dashed box, and the protected sequence is shown at the bottom. The palindromic sequence in the protected region is shown in black box. (B) McbG binding to the promoter DNA with the 25-bp motif deleted. The first three lanes are wild-type *mcbBCDEF* promoter DNA, which was used as the control, and the next three lanes are 25-bp motif-deleted DNA probes. The sample in each lane was incubated with 0.05 mM 1-naphthol. (C) Electrophoretic mobility shift assays of McbG binding to the mutant *mcbBCDEF* cluster promoter DNA. The nucleotide sequence 5′-TATCGATA-3′ in the McbG-binding site was mutated to 5′-TTTAACCC-3′ (mutant promoter 1), and the nucleotide sequence 5′-CCTTTAAGCATTGGTAT-3′ in the McbG-binding site was mutated to 5′-AGGTAAGGTAAGGTAAT-3′ (mutant promoter 2). The first 3 lanes show wild-type *mcbBCDEF* promoter DNA, which was used as the positive control, the middle 3 lanes contain mutant promoter 1 DNA, and the last 3 lanes contain mutant promoter 2 DNA. The sample in each lane was incubated with 0.05 mM 1-naphthol.

sequence is located upstream of the *nod* gene between 75 and 20 bp and is known as Nod-box (29). The LysR-type transcriptional regulator (LTTR) box containing T-N_{11}-A at the ribosome binding site was originally discovered in *Pseudomonas putida* PRS3026 (30). This structure is present in LysR family regulators, including DbdR, involved in the anaerobic degradation of 3,5-dihydroxybenzoate in *Thauera aromatica* AR-1 (31), BenM, which catalyzes degradation of benzoate in *Acinetobacter baylyi* ADP1 (32), and

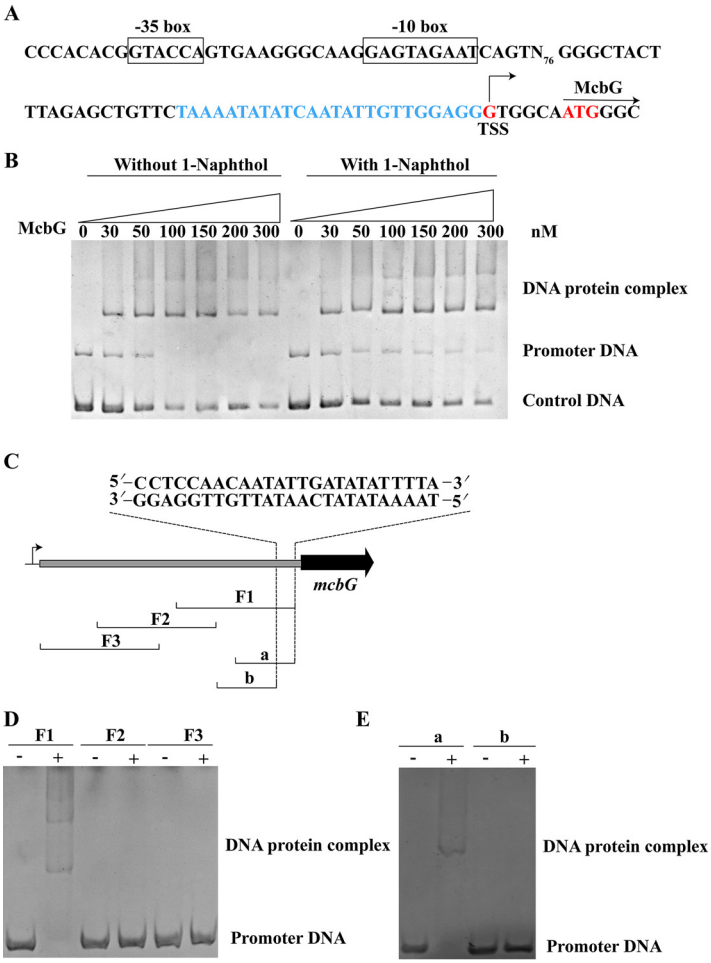

FIG 7 (A) DNA elements in the promoter of *mcbG*. The −35 and −10 boxes are shown in boxes, and the TSS is shown by an arrow. The McbG-binding site is indicated by blue sequence. (B) Electrophoretic mobility shift assays on the binding of McbG to the *mcbG* promoter. Each lane contains 20 ng of DNA probe. The first 7 lanes show samples incubated without 1-naphthol, and the next 7 lanes show samples incubated with 0.05 mM 1-naphthol. The concentrations of McbG, increasing from left to right, are shown above the lanes. The control DNA was a 200-bp fragment that was amplified from the *mcbC* gene. (C) Schematic diagram of the *mcbG* promoter-*mcbG* intergenic region and the DNA subfragments used to determine the McbG binding site. The locations of fragments used in the EMSAs are shown below. The sequence at the top shows the McbG binding site. (D and E) EMSAs of subfragments F1, F2, and F3 (D) and a and b (E) with purified McbG. The lanes contain the following: the DNA fragment (20 ng) alone (−) and the DNA fragment (20 ng) with McbG (200 nM) (+).

DntR of *Burkholderia* sp. strain DNT, which is involved in the degradation of 2,4-dinitrotoluene (33). An LTTR box (T-N_{11}-A) was also found in the McbG binding site, concurrent with a specific palindrome sequence, 5′-TATCGATA-3′, inside the box. This short palindromic sequence of T-N_6-A at the McbG binding site has been proven to be important for binding of the protein to DNA (Fig. 6C). In addition, the amino acid sequences of McbG in strains XWY-1 and C5pp shared 100% identity, and the binding site of McbG in the *mcbBCDEF* promoter sequence area of strain XWY-1 is also present in the *mcbBCDEF* promoter sequence area of strain C5pp (Fig. S5). Therefore, we speculate that the regulation mechanism of McbG is the same in these two strains.

In this study, it was also found that McbG regulated itself. This self-regulation phenomenon is common in the LysR-type regulator family. IlvY activated the expression of acetohydroxy-acid isomeroreductase gene *ilvC* in *E. coli* and *Salmonella* spp. and negatively regulated it by itself (34, 35). The CidR involved in regulating the transcription of the *cidABC* gene cluster responsible for cell death in *Staphylococcus aureus* and *Bacillus anthracis* also has its own negative regulation (36, 37). The GltC that regulates the

expression of the glutamate synthase gene *gltAB* in *B. subtilis* can also repress its own expression (38). In strain XWY-1, the transcription level of *mcbG* was significantly upregulated in the presence of 1-naphthol (Fig. S4), indicating that 1-naphthol induced the transcription of *mcbG*. The EMSA results showed that McbG can bind to its own promoter, while the addition of 1-naphthol weakens this binding (Fig. 7B). This result suggested the McbG may mediate the transcription of *mcbG*.

In strain XWY-1, *mcbABCDEF* is responsible for the upstream pathway of the degradation of carbaryl (from carbaryl to salicylate), with the *mcbA* gene being constitutively expressed (20). The present study proved that *mcbBCDEF* is a transcriptional unit and that McbG is its activator. To test whether McbG is involved in the regulation of *mcbIJKL* and *mcbOPQ*, which are responsible for midstream and downstream pathways of carbaryl metabolism, the transcription of *mcbBCDEF*, *mcbIJKL*, and *mcbOPQ* was analyzed in strains XWY-1, MT, and MTC. The results showed that the transcription of the genes in the *mcbBCDEF*, *mcbIJKL*, and *mcbOPQ* clusters was not significantly different in strain MT with or without the induction of 1-naphthol (Fig. 4, Fig. S6A). When salicylate was used as an inducer, the transcription of the genes in the *mcbBCDEF* cluster was not significantly altered in strain MT. However, transcription of the *mcbIJKL* and *mcbOPQ* gene clusters was similar to that of strain XWY-1 and the complemented strain MTC (Fig. S6B), indicating that salicylate can be further degraded as a substrate in the midstream and downstream pathways, thus triggering transcription of the *mcbIJKL* and *mcbOPQ* gene clusters. These results indicate that *mcbG* is a transcriptional activator of the *mcbBCDEF* cluster but is not responsible for the regulation of the *mcbIJKL* and *mcbOPQ* gene clusters of carbaryl metabolism. Therefore, further studies are needed to elucidate the regulatory mechanisms of the midstream and downstream pathways of the carbaryl metabolism.

MATERIALS AND METHODS

Chemicals and media. 1-Naphthol (98% purity), purchased from Shenzhen Sendi Biotechnology Co. Ltd. (Shenzhen, China), was prepared as a 0.4 g liter^{-1} stock solution in water and was sterilized by membrane filtration (pore size, 0.22 μm). MSM consisted of the following components (g liter^{-1}): 1.0 NH_4NO_3, 1.0 NaCl, 1.5 K_2HPO_4, 0.5 KH_2PO_4, and 0.2 $MgSO_4 \cdot 7H_2O$, pH 7.0; the carbon source was added as required. Glucose medium (GM) was MSM supplemented with 1% glucose (wt/vol) as the sole carbon source. Luria-Bertani (LB) broth consisted of the following components (g liter^{-1}): 10.0 tryptone, 5.0 yeast extract, and 10.0 NaCl at pH 7.0.

Bacterial strains, oligonucleotides, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1, and the oligonucleotide primer sequences are listed in Table 2. Strain XWY-1 (deposited in the China Center for Type Culture Collection [CCTCC]; accession number AB2020137) is a carbaryl-degrading strain that was previously isolated in our lab. *E. coli* strains were used for recombinant DNA procedures and were grown at 37°C in LB medium or LB medium supplemented with antibiotics as described. Other bacterial strains were grown aerobically at 30°C in LB broth or LB agar. Expression of 1-naphthol metabolic genes was induced in MSM supplemented with 0.1 mM 1-naphthol. Chloramphenicol (Cm) and tetracycline (Tc) were used at 30 μg ml^{-1}, kanamycin (Km) and gentamicin (Gm) were used at 50 μg ml^{-1}, and ampicillin (Amp) was used at 100 μg ml^{-1}. Growth of cells was evaluated by measuring the optical density at 600 nm (OD_{600}).

Determination of the transcription start sites. The transcription start sites of the *mcbBCDEF* cluster were determined using a 5′ rapid amplification of cDNA ends (RACE) system (TIANDZ; Beijing Tianenze Gene Technology Co. Ltd.). First-strand cDNA was synthesized using the primer SP1 (Table 2), and tailing of the cDNA was performed using terminal transferase and dTTP. The deoxyribosylthymine (dT)-tailed cDNA was amplified using the abridged anchor primer (APP) and SP2 (Table 2). This PCR product was then used as a template for a nested PCR with an abridged universal amplification primer (AAP) and primer SP3 (Table 2) and cloned into a pMD19-T vector (TaKaRa, Japan) for sequencing.

Genetic disruption and complementation. Two DNA fragments corresponding to 1,000-bp upstream and downstream flanking regions of the *mcbG* gene were amplified using primer pairs McbGupF/McbGupR and McbGdownF/McbGdownR, respectively. They were linked to a kanamycin resistance gene that was amplified from plasmid pBBR1MCS-2 (39) with the primer pair McbGkF/McbGkR by overlap extension PCR, and the resulting fragment was cloned into pEX18-Gm (40), yielding pEX*mcbG*. pEX*mcbG* was then electroporated into strain XWY-1. Single-crossover mutants were screened on LB agar containing 50 μg ml^{-1} of kanamycin and 50 μg ml^{-1} of gentamicin. After verification, a single-crossover mutant was cultured until the OD_{600} reached approximately 0.2, and double-crossover mutants were selected on LB agar containing 50 μg ml^{-1} of kanamycin and 20% sucrose. Both single- and double-crossover mutants were verified by PCR and DNA sequencing. The double-crossover mutant was designated MT. The *mcbC* knockout mutant XWY-1 Δ*mcbC* was obtained similarly.

The *mcbG* gene was amplified with the primer pair McbGhbF/McbGhbR inserted into pBBR1MCS-5 (39) to generate pB*mcbG*, which was electroporated into MT to obtain the *mcbG*-complemented strain MTC.

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Characteristic(s)[a]	Source or reference
E. coli strains		
DH5α	F$^-$ *recA1 endA1 thi-1 supE44 relA1 deoR*Δ*(lacZYA-argF)U169 80dlacZ*Δ*M15*	TaKaRa
BL21(DE3)	F$^-$ *ompT hsdS*(r_B^- m_B^-) *gal dcm lacY1* (DE3)	TaKaRa
Pseudomonas sp. strains		
XWY-1	Carbaryl degradation strain, Amp^r; Cm^r	Lab stock
MT	*mcbG*-disrupted mutant from strain XWY-1; Amp^r; Km^r	This study
MTC	MT harboring pB*mcbG*; Gm^r; Amp^r; Km^r	This study
XWY-1 Δ*mcbC*	*mcbC*-disrupted mutant from strain XWY-1; Amp^r; Gm^r	This study
Plasmids		
pMD19-T	TA cloning vector, Amp^r	TaKaRa
F1-19T	300-bp fragment, upstream of TSS of *mcbBCDEF*, directionally cloned into pMD19-T, Amp^r	This study
pBBR1MCS-2	Broad-host-range cloning vector, Km^r	39
pBBR1MCS-5	Broad-host-range cloning vector, Gm^r	39
pEX18Gm	Gene knockout vector, *oriT*, *sacB*, Gm^r	40
pEX*mcbG*	*mcbG* gene knockout vector containing upstream and downstream homologous regions of *mcbG*, Gm^r	This study
pB*mcbG*	pBBR1MCS-5 harboring *mcbG*, Km^r	This study
pET-29a(+)	Expression vector, Km^r	Novagen
pET-*mcbG*	pET-29a(+) harboring *mcbG*, Km^r	This study
pME6522	pVS1-p15A *E. coli-Pseudomonas* shuttle vector for transcriptional *lacZ* fusion and promoter probing, Tc^r	21
pME6522-P_{mcb}	300-bp fragment, upstream of TSS of *mcbBCDEF*, directionally cloned into pME6522, Tc^r	This study

[a]Cm^r, chloramphenicol resistant; Amp^r, ampicillin resistant; Km^r, kanamycin resistant; Gm^r, gentamicin resistant; Tc^r, tetracycline resistant.

Quantitative RT-PCR (qRT-PCR). Strains XWY-1, MT, and MTC were cultured in LB medium with appropriate antibiotics to an OD_{600} of 0.6. The cells were then harvested and washed twice with MSM. Expression of 1-naphthol degradation genes in washed cells was induced in MSM (the final cell density corresponded to an OD_{600} of 2.0) at 30°C for 3 h in the presence of 0.1 mM 1-naphthol. A culture grown in MSM with 0.1 mM glucose was used as the control. Total RNA was extracted using total a RNA extraction kit (TaKaRa, China), and genomic DNA (gDNA) in the preparation was digested with gDNA Eraser (TaKaRa, China) at 42°C for 2 min. Reverse transcription was then performed with 1 μg of gDNA-free RNA using random primers. The cDNA was synthesized by incubation at 37°C for 15 min using PrimeScript reverse transcriptase (RTase, TaKaRa), and the reaction was stopped by heating the mixture at 85°C for 5 s. Samples were diluted 100-fold to serve as templates for quantitative PCR (qPCR). qPCR was performed in an Applied Biosystems 6 real-time PCR system using a SYBR premix *Ex Taq* RT-PCR kit (TliRNaseH Plus; TaKaRa, China) per the manufacturer's instructions. The 16S rRNA gene was used as an internal standard, and relative expression was quantified using the $2^{-\Delta\Delta CT}$ threshold cycle (C_T) method.

Reporter plasmid construction and β-galactosidase activity assay. The P_{Mcb}, a 300-bp fragment upstream of the TSS of *mcbBCDEF*, was amplified by PCR from strain XWY-1 using the oligonucleotide pair mcb-bF/mcb-bR (Table 2). The PCR product was digested with EcoRI and PstI, followed by ligation into pME6522 to generate pME6522-P_{Mcb} carrying the P_{Mcb}-*lacZ* transcriptional fusion (Table 1).

β-Galactosidase activity assays were performed with strain XWY-1 grown in GM or in GM supplemented with 0.1 mM 1-naphthol. β-Galactosidase activity was determined using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate. The observed activity was normalized to the optical density of the culture at 600 nm and expressed in Miller units (41). One Miller unit of enzyme activity is defined as the amount of enzyme required to catalyze ONPG to produce 1 μmol *o*-nitrophenol (ONP) per minute. At least three independent cultures from each strain were assayed in each experiment.

Purification of McbG and EMSA. To determine the function of McbG, the *mcbG* gene was expressed in *E. coli* BL21(DE3), and the protein was purified as described by Ni et al. (42). The *mcbG* DNA fragment was amplified with the primers mcbGbdF/mcbGbdR, digested with XhoI and NdeI, and inserted into similarly cut pET-29a(+) to produce pET-*mcbG*. The clones were sequenced to ensure that no mutations were introduced. *E. coli* BL21(DE3) harboring pET-*mcbG* was grown in LB at 37°C to an OD_{600} of 0.6 to 0.8 and then induced for 12 h by the addition of 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 16°C. Cells were then collected, washed, and disrupted by sonication. After centrifugation at 12,000 × *g* for 30 min, the supernatant was precipitated using 40% ammonium sulfate, followed by centrifugation at 12,000 × *g* for 20 min. The precipitate was dissolved in Tris-HCl and loaded onto a His-Bind resin. His8-tagged McbG was eluted using 50 mM Tris-HCl buffer with various concentrations of imidazole (0 mM, 25 mM, 50 mM, 300 mM, and 500 mM). Fractions containing McbG were pooled and filtered using a 10-kDa Amicon ultrafiltration tube. Protein concentration was determined using the Bradford method. The molecular mass of the purified enzymes was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

For the EMSA, a nonradioactive strategy was implemented according to the method described by De la Cruz et al. (43). The wild-type 300-bp promoter DNA probe of the *mcbBCDEF* cluster was amplified using mcb-F/mcb-R; the 300-bp sequence containing the promoter area of *mcbG* was amplified using

TABLE 2 Oligonucleotides used in this study

Function and oligonucleotide	Sequence (5′→3′) [a]
Gene disruption	
McbGupF	TTCCCGTTGAATATGGCTCATCAGATCATCTTTAAAAATACCCCTCAGTTG
McbGupR	TATGACCATGATTACGAATTCCAGTTCAGCCTGCTGTTCATT
McbGdownF	CAGGTCGACTCTAGAGGATCCGCGCGACATGCTTGAACT
McbGdownR	ATGCTCGATGAGTTTTTCTAAGAGGGCACGGACCTTATATCG
McbGkF	CGATATAAGGTCCGTGCCCTCCGATATAAGGTCCGTGCCCTC
McbGkR	GGTATTTTTAAAGATGATCTGGGTATTTTTAAAGATGATCTG
McbGhbF	CGCTCTAGAACTAGTGGATCCGACGACGCAGGCAGCAC
McbGhbR	GATAAGCTTGATATCGAATTCTCAGACCTTCCTTAAGGTATCTCTTAACC
McbCDQ-F	CAGGTCGACTCTAGAGGATCCCCCGGGGAAAAAATCCCAGC
McbCDQ-R	TATGACCATGATTACGAATTCGAGTTCGAAGGAAAAATGGGAG
Transcriptional unit analysis	
McbAB-F	TAACTATTTTGTGAGTTGGTTGG
McbAB-R	CCTGCATTACCTTCACATC
McbBC-F	GTGCGTCCAGATCGTTTTGTG
McbBC-R	GACACAGCCCAATCAACCCCA
McbCD-F	CCAGCAATCGAGAGCTTCCTG
McbCD-R	GACTGCTCGATTTCCAGAACTTC
McbDE-F	GAGAGGTGATGAGGTTGGGTG
McbDE-R	GGATGAACCTCAAGTCAATTCGAT
McbEF-F	CTTCCTGTGTCATCGGTCCTA
McbEF-R	GCAACATCGCGATAAAACTGG
McbFR-F	CGTCGACACAGCAGTGCTATG
McbFR-R	GCGTGAGTCTTCGGACTAGTC
qRT-PCR analysis	
16Srt-F	GTAGATATAGGAAGGAACACCAGTGG
16Srt-R	TTAACCTTGCGGCCGTACTC
mcbBrt-F	GGCTTGGGATCAAAAGGGATG
mcbBrt-R	GCCGAATATTGCAACCCGTTC
mcbCrt-F	GCCGATATTGCTGGATGTTGC
mcbCrt-R	ACCAAGCTACGAGCACCAT
mcbDrt-F	CCAGCAATCGAGAGCTTCCTG
mcbDrt-R	CGCCCCTTAGGAGACTCAATG
mcbErt-F	GAGAGGTGATGAGGTTGGGTG
mcbErt-R	GCTCGAAGCCAAACGCAAATC
mcbFrt-F	GGCGAATGAAGTTGGTTCCTC
mcbFrt-R	GCGTGAGTCTTCGGACTAGTC
mcbGrt-F	TATCTGGATGTGCTGTTTCAGCC
mcbGrt-R	AGGAACCTCTGCCAAAGAGCTA
mcbIrt-F	TTCAAAGCCTCGACCATGGC
mcbIrt-R	TGTTGTCGATTGTGCGAGGTG
mcbJrt-F	ATTGACCTTGCCGTCCTGC
mcbJrt-R	CTTGAGCGCCTGTTTTACTCAG
mcbKrt-F	AAGCTTAAGCCCTTCCGGAG
mcbKrt-R	AGAACTATGAGCGCGGGT
mcbLrt-F	TCACCGGGTAGGTCCGGA
mcbLrt-R	ATGGGTCGATGTTGCACC
mcbOrt-F	CCCGATGGTGCGCTTCTT
mcbOrt-R	GTCTTGGCACAGCAGGGTTT
mcbPrt-F	GACTACCCGGTGGAGACCAG
mcbPrt-R	GTCGAAGGCCTTGCCCAG
mcbQrt-F	CGCCCAGCTCCCGTTGTA
mcbQrt-R	GTAGTCTTCAAGCTCGCAGCC
Gene expression	
mcbGbdF	TCAGTGGTGGTGGTGGTGGTGGTGCTCGAGGACCTTCCTTAAGGTATCTCT
mcbGbdR	TAAGAAGGAGATATACATATGATGGGCTATAAAAACAGATCC
DNA affinity and EMSA	
mcb-F	CTGTAGGGCAGCCCAGCG
mcb-R	GCAACACACCCATGCACCAC
mcbC-F	GCCGATATTGCTGGATGTTGC
mcbC-R	CCTTCGAACTCAAACCCGAGG

(Continued on next page)

TABLE 2 (Continued)

Function and oligonucleotide	Sequence (5′→3′) [a]
JHQ1-F	ACCAGCCGAAGCATCAACTTG
JHQ1-R	GCAGGCAGTGTTTTTCAGCCCTGTTGGTATTGAAAAGCACTGTTACTCA
JHQ2-F	CAGTGCTTTTCAATACCAACAGGGCTGAAAAAACACTGCCTG
JHQ2-R	TCGAGACACAGGCATGGTGT
DTB-1R	GTGTTTTTCAGCCCCCTTTAAGCATTGGTATTTTAACCCTGTTGGTATT
DTB-2R	AATACCAACAGGGTTAAAATACCAATGCTTA AAGGGGGCTGAAAAACACT
DTB-3R	GGGGCAGGCAGTGTTTTTCAGCCCAGGTAAGGTAAGGTAATTATCGATA
DTB-4R	ACATATCGATAATTACCTTACCTTACCTGGGCTGAAAAACACTGCCTGCCC
mcbG-F	TGCCACCTCCAACAATATTGATATATT
mcbG-R	CGAAACCGTGTTTGAATACATCG
GF1-F	CCTCCAACAATATTGATATATTTTAG
GF1-R	CACGGTACCAGTGAAGGGC
GF2-F	GGCTATTTCCGCGCTACTG
GF2-R	AGAGGTCTCCGGGGCCCG
GF3-F	TGGGCTGCTGTCCGCATTA
GF3-R	AATCGACTACAACCGCCAG
Ga-R	GCTTATTGGACGAAACGAAGGAAA
Gb-F	GAACAGCTCTAAAGTAGCCCCG
Gb-R	TGACGCAGGGGACCTTCG
Reporter plasmid construction	
mcb-bF	**CGGAATTCC**TGTAGGGCAGCCCAGCG
mcb-bR	**AACTGCAG**GCAACACACCCATGCACCAC
5′ RACE	
SP1	CCGGTAAAATTAACCCTCCGC
SP2	CGATTAGCGCGTGAGTCTTC
SP3	GGCTTGTCGGTCGGAATAGTC

[a]Restriction sites are in boldface, and nucleotide sequences that are different from the template are underlined.

mcbG-F/mcbG-R; a 200-bp region of *mcbC* used as the negative control was amplified using the primer mcbC-F/mcbC-R. Nucleotides in the mutated promoter DNA were introduced by primers (listed in Table 2) and were amplified using overlapping extension PCR. The *mcbBCDEF* fragment with the deleted 25-bp motif sequence (CCTTTAAGCATTGGTATTATCGATA) was amplified with overlap extension PCR using primers JHQ1-F/JHQ1-R and JHQ2-F/JHQ2-R. The *mcbG* promoter subfragments F1, F2, F3, a, and b were amplified using primers GF1-F/GF1-R, GF2-F/GF2-R, GF3-F/GF3-R, GF1-F/Ga-R, and Gb-F/Gb-R (listed in Table 2). Approximately 20 ng of a promoter probe was mixed with increasing concentrations of purified McbG in a binding buffer (100 mM Tris-HCl [pH 8.0], 50 mM KCl, 5% [vol/vol] glycerol, 250 μg ml^{-1} EDTA, and 1 mM dithiothreitol [DTT]). A nonspecific DNA sequence (a sequence in *mcbC*) was used as the negative control. The effects of 1-naphthol on the binding of McbG to the promoter probes were evaluated by adding 0.05 mM 1-naphthol to the reaction system. The mixture was incubated at 25°C for 30 min and then separated on a 5% (vol/vol) native polyacrylamide gel electrophoresis in 0.5× Tris-glycine-EDTA. The DNA and DNA-protein complexes were visualized using ethidium bromide staining.

DNase I footprinting assay. For preparation of fluorescent 6-carboxyfluorescein (FAM)-labeled probes, the promoter region was amplified by PCR using 2× TOLO HIFI DNA polymerase premix (TOLO Biotech, Shanghai, China) from the plasmid F1-19T using the primers M13F(FAM) and M13R. The FAM-labeled probes were purified using the Wizard SV gel and PCR clean-up system (Promega, USA) and were quantified using NanoDrop 2000C (Thermo, USA).

DNase I footprinting assays were performed similarly to the description by Wang et al. (44). For each assay, 350-ng probes were incubated with different concentrations of McbG in a total volume of 40 μl. After incubation for 30 min at 25°C, a 10-μl solution containing about 0.015 units DNase I (Promega) and 100 nmol freshly prepared $CaCl_2$ was added and incubated at 37°C for 1 min. The reaction was stopped by adding 140 μl DNase I stop solution (200 mM unbuffered sodium acetate, 30 mM EDTA, and 0.15% SDS). Samples were first extracted with phenol-chloroform (1:1, vol/vol) and then precipitated with absolute ethanol. The pellets obtained were dissolved in 30 μl Milli-Q water. Preparation of the DNA ladder, electrophoresis, and data analysis were as described earlier (44), except that the GeneScan-LIZ600 size standard (Applied Biosystems) was used.

Analytical techniques. To analyze 1-naphthol, the culture samples were centrifuged at 12,000 × *g* for 5 min. The supernatants were filtered through 0.22-μm-pore-size filters before being subjected to analysis with a high-performance liquid chromatography (HPLC) system (UltiMate 3000; Dionex, USA) equipped with a C_{18} reverse-phase column (4.6 by 250 mm, 5 μm). The mobile phase consisted of methanol-water-acetic acid (75:25:0.5, vol/vol/vol) at a flow rate of 1 ml min^{-1} at 40°C for 10 min. Column elution was monitored by measuring the absorbance at 230 nm.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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