JOURNAL OF BACTERIOLOGY, Jan. 2006, p. 456–463 Vol. 188, No. 2
0021-9193/06/$08.00+0 doi:10.1128/JB.188.2.456–463.2006

Expression Analysis of Up-Regulated Genes Responding to Plumbagin in *Escherichia coli*

Jenn-Wei Chen,[1,4] Chang-Ming Sun,[2,3] Wei-Lun Sheng,[1] Yu-Chen Wang,[1] and Wan-Jr Syu[1]*

Institute of Microbiology and Immunology[1] and Institute of Biochemistry and Molecular Biology,[2] National Yang-Ming University,
National Research Institute of Chinese Medicine,[3] and Department of Research and Development,
U-Vision Biotech Inc.,[4] Taipei, Taiwan, Republic of China

Received 14 June 2005/Accepted 25 October 2005

Plumbagin is found in many medicinal plants and has been reported to have antimicrobial activities. We examined the molecular responses of *Escherichia coli* to plumbagin by using a proteomic approach to search for bacterial genes up-regulated by the drug. The protein profile obtained was compared with that of *E. coli* without the plumbagin treatment. Subsequent analyses of the induced proteins by mass spectroscopy identified several up-regulated genes, including *ygfZ*, whose function has not been defined. Analyses of the 5′-flanking sequences indicate that most of these genes contain a marbox-like stretch, and several of them are categorized as members of the *mar*/*sox* regulon. Representatives of these genes were cloned into plasmids, and the marbox-like sequences were modified by site-directed mutagenesis. It was proven that mutations in these regions substantially repressed the level of proteins encoded by the downstream genes. Furthermore, plumbagin's early effect was demonstrated to robustly induce SoxS rather than MarA, an observation distinctly different from that seen with sodium salicylate.

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) is a naphthoquinone having antibacterial (6), antifungal (5), anticancer (22), and antimutagenic activities (7). Like other redox-cycling chemicals such as paraquat and menadione, plumbagin has been used as an agent to generate superoxide or reactive oxygen species in order to study oxidative stress (14). Plumbagin has been suggested to activate SoxS by oxidizing the SoxR molecule (10, 21) or inhibiting the repression of MarR (2), the effect of which is exerted on *marA*, resulting in activation of the *mar*/*sox* regulon. In addition, it has been observed that the expression of some members of the *mar*/*sox* regulon in *Escherichia coli*, such as *sodA* (8), *nfo* (3), *ribA* (20), and *pqi* (21), is up-regulated by treatment with plumbagin.

Although plumbagin could induce excessive expression of superoxide dismutase and catalase, overexpression of *sodA* failed to protect *E. coli* (17). The toxic effect of plumbagin may not simply result from the production of reactive oxygen species. It has been reported that plumbagin inhibits NADH dehydrogenase, as well as causing respiratory arrest (17). Plumbagin has also been shown to modify the lactose carrier and inhibit its binding with galactoside; the modified carrier then becomes completely inactive (29). The above effects appeared more or less to result directly from the chemical nature of plumbagin. In this report, we focus on the responses of the bacteria to the chemical, in which multiple proteins were simultaneously induced by plumbagin treatment. We report the evaluation of the bacterial regulatory systems after treating *E. coli* with plumbagin.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *E. coli* JM109 was used as the major experimental strain for the chemical treatments and the cloning host. Plasmids pGEM-T-easy (Promega, Madison, WI), pBluescript II SK+ (Stratagene, La Jolla, CA), and pQE60 (QIAGEN, Valencia, CA) were used as general cloning vectors. Plasmid pMH was modified from pQE60 by deleting the T5 promoter. Bacteria were cultivated in tubes containing Luria-Bertani medium. To prepare proteins for two-dimensional gel electrophoresis (2-DE), bacteria (80 ml) were grown at 37°C with vigorous shaking in 250-ml flasks. To measure the MIC, either an agar diffusion assay or the liquid broth method was used and the standard procedure recommended by the Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards) was followed. Plumbagin (Sigma, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) or methanol with a stock concentration at 10 mg/ml without further purification.

2-DE. Bacteria from overnight cultures were diluted 100-fold into 80 ml of Luria-Bertani medium. Bacteria were grown with aeration at 37°C to an A_{600} of 0.2. Plumbagin was added to make a final concentration of 25 μg/ml (0.13 mM), and the culture was further agitated at 37°C for 2 h. Protein extraction was performed as previously described (12), except for a slight modification. In brief, bacteria were harvested by centrifugation and washed twice with cold 0.9% NaCl. After resuspension in 3 ml of TSD buffer (28 mM Tris HCl, 22 mM Tris, 0.3% sodium dodecyl sulfate, 200 mM dithiothreitol) and addition of 0.3 g of glass beads (0.1 to 0.25 mm in diameter), the bacteria were broken by vigorous vortexing for 10 min. Unbroken bacteria were removed by centrifugation, and the supernatant was boiled for 5 min. Chilled samples received 150 μl of TM buffer (24 mM Tris, 476 mM Tris HCl, 50 mM $MgCl_2$) containing DNase I (1 mg/ml) and RNase A (0.25 mg/ml). The enzymatic digestion was stopped after 30 min by adding 4 volumes of ice-cold acetone, and samples were incubated overnight at −20°C. Proteins were collected by centrifugation at 11,000 × *g* for 15 min and then dissolved in 200 μl of isoelectric focusing (IEF) sample buffer containing 8 M urea, 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), and 40 mM Tris base. The first-dimension (1-D) IEF was performed with the Ettan IPGphor II IEF System, and 2-DE was carried out with an SE260 electrophoresis apparatus (Amersham Biosciences, Piscataway, NJ).

Western blotting. The bacterial culture and plumbagin treatment were similar to those used for 2-DE. The proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane. To detect ectopically expressed His_6-tagged proteins, a rabbit polyclonal antibody specific for the tag (Bethyl, Montgomery, TX) was used as the primary antibody. A horseradish peroxidase-conjugated goat anti-rabbit antibody (Sigma) was used as the secondary antibody. The membranes were finally developed by a chemiluminescence method (16). The images devel-

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* Corresponding author. Mailing address: Institute of Microbiology and Immunology, National Yang-Ming University, 155 Sec. 2, Li-Nong Street, Beitou, Taipei 112, Taiwan. Phone: 886 2 28267112. Fax: 886 2 28212880. E-mail: wjsyu@ym.edu.tw.

oped on films were quantitatively monitored with Personal Densitometer SI (Molecular Dynamics).

Plasmid construction. To construct pMH-mdaB, a 1,590-bp fragment covering the entire *mdaB* gene and its 5′-flanking region was obtained by PCR amplification with the genomic DNA of *E. coli* as the template and primers NmdabF (CGGGATCCCCGC TATTGCTAAGTTTG) and NmdabR (AACTGCAGTTTCCACAAGATGCTTGCG). The PCR product was digested with BamHI/PstI and ligated into pMH that had been previously digested with the same enzymes. The resulting plasmid was named pMH-mdaB, and the encoded MdaB protein had a hexahistidine extension at the C terminus. Plasmids pMH-ahpC, pMH-gatY, pMH-nfnB, pMH-nfo, pMH-tpx, pMH-talB, pMH-ygfZ, pMH-ygfZ′, pMH-ygaG, pMH-marRA, and pMH-soxRS were similarly generated by the same strategy, except that the primer pairs used were NahpcF (CGGGATCCT CGTCCATCAGTTTCTC)-NahpcR (AAGTCGACTTTACCAACCAGGT CC), NgatyF (CGGGATCCTTATGCGCATCGCATTC)-NgatyR (AACTGCA GCACAATCGAATCAC), NnfnbF (GCGGATCCTGGCCCTTGAGTTAC CC)-NnfnbR (GCGGATCCATTCGTTAGGTGATG), NnfoF (CAGGATCCA CATCGTACACTGTGGC)-NnfoR (GGGAGGCTACCGCTTTTTCAG), NtpxF (CCCGGATCCTAAAGATGCAATTCGC C)-NtpxR (CCACTGCAGG TGCTTTCAGTACAGCC), NtalbF (TCCCGGGCTGCCAGCCAAAAGCA)-NtalbR (GGAAGATCTCAGCAGATCGCCGATC), NygfzF (GCGGATCCC AAACGCCCGTCCATCAACAAGCG)-NygfzR (GCGGATCCGGCGGTGG ATCTTAGCCAGCGCC), Nygfz′F (CGGGATCCCGAAATTAATTCAC)-NygfzR, NygagF (GCGTCGAAGTGCGTTCGCTGGACATCAACCCG)-NygagR (GCGGATCCGGATATGCGGCTGGTAACCTTC), NmarraF (GGGCTGCAGGCCAATTGCTTTAAACAAATC)-NmarraR (GGGAGATCTGCTGTTGTAATGATTTAATGG), and NsoxrsF (CCTCTGCAGGCCCTTGTGGCGCTTTAG)-NsoxrsR (CCTAGATCTCAGGCGGTGGCGATAATCG), respectively.

To construct pACYC-soxRS, which expresses SoxS from a plasmid compatible with pMH, the *soxRS*-containing fragment was obtained by treating pMH-soxRS with BamHI and HindIII and cloned into pACYC184 that had been digested with the same enzymes.

Site-directed mutagenesis. The 5′-flanking region of *mdaB* was PCR amplified in a similar way from *E. coli* genomic DNA with primer NmdabF paired with NmdabMR (CAAAATGCGCAAAGTCTTTTG) or NmdabMF (CAAAAGAC TTTGCGCATTTTG) paired with NmdabR. The two PCR products separately amplified were mixed. Since NmdabMR and NmdabMF embedding the designated point mutations are complementary in sequence, the two DNA products were annealed at one end and extended by Vent polymerase and deoxynucleoside triphosphates. After 10 reaction cycles, NmdabF and NmdabR were added to the mixture and PCR amplification was continued for an additional 30 cycles. The PCR product was then digested with BamHI and PstI and cloned into pMH, resulting in plasmid pMH-mdaBm. Mutation-bearing plasmids pMH-nfom and pMH-ygfZ′m were constructed by a similar strategy; the paired primers used accordingly for the mutagenesis were NnfoMF (GTGATTCAAAGCGTCATTCT AGAAACCAC)-NnfoMR (GCAAGATGTAGTGGTTTCTAGAATGACGC) and NygfzMF (GTCGCCTCGCAGAAGGTACCGATCGCGTAGTG)-NygfzMR (AGTGCTTTCACTACGCGATCGGTACCTTCTGCG), respectively.

Q-TOF mass spectroscopy. In-gel tryptic digestion was performed as previously described (30), except for a slight modification. In brief, Coomassie blue dye-stained protein spots excised from a distilled-water-washed gel were cut into small pieces. Gel pieces were dehydrated and destained with three washes with 50 mM ammonium bicarbonate mixed with 50% (vol/vol) acetonitrile. After dehydration and in-gel digestion with trypsin, the peptides recovered were analyzed with a quadrupole time-of-flight (Q-TOF) mass spectrometer (Waters, Milford, MA). Data from the mass profiling were used to search the NR database with Mascot (http://www.matrix-science.com).

Construction of a *soxS* mutant strain. To delete *soxS* from the chromosome by homologous recombination (4), *E. coli* strain JM110 was used as the parental strain since strain JM109 lacks *recA* and has a low frequency of recombination. To perform the deletion, both the 5′- and 3′-flanking regions of *soxS* were PCR amplified from *E. coli* genomic DNA. The primer pairs used were 5′NsoxSF (GCGGATCCGCATCAACACCAACCGGAACC)-5′NsoxSR (GCGAATTCG TCAATCCATGCGATAAGATCC) and 3′NsoxSF (GCGAATTCCTGTAATT TTATTGCCCGCGCG)-3′NsoxSR (GCGGATCCTTCGACAAAACGCCCA ATTGC), respectively. The PCR products were then cloned into pGEM-T Easy and confirmed after sequencing. These fragments were then excised from the plasmids with BamHI/EcoRI and three-way ligated with BamHI-restricted pKO3 (25), yielding pKO3-ΔsoxS. pKO3-ΔsoxS was then transformed into strain JM110, and the resulting transformants were sequentially selected in chloramphenicol- and sucrose-containing media. The *soxS* mutant strain obtained was confirmed by PCR amplification of the region spanning the target site, followed by restriction enzyme analysis and sequence confirmation.

RESULTS

Proteins whose expression is up-regulated in *E. coli* by plumbagin. To investigate the response of the bacteria, *E. coli* was treated with plumbagin at a subinhibitory concentration. Plumbagin was first determined to have an MIC against *E. coli* JM109 of 50 μg/ml. Thus, the plumbagin treatment was held at 25 μg/ml for 2 h and the total bacterial proteins were extracted and separated by 2-DE. Proteins on the gel were stained with Coomassie blue dye and compared with those prepared from bacteria without plumbagin treatment. Twelve protein spots whose expression was obviously increased by plumbagin treatment (Fig. 1) were cut out and digested with trypsin. The tryptic peptides eluted had their amino acid sequences deduced by Q-TOF mass spectroscopy. The peptides so deduced were used to search for matching proteins derived from the *E. coli* genome. Figure 2A shows a typical result of three tryptic peptides matched to the *E. coli* alkyl hydroperoxide reductase (AhpC). Results from similar identifications are listed in Fig. 2B.

Plumbagin-responding genes. Among the 12 deduced proteins, SodA was massively induced by plumbagin and could simply be identified by 1-D SDS-PAGE, followed by Coomassie blue dye staining. Among the remaining 11 protein spots, although the 2-D gel profiling of the proteins was reproducible, misleading identification could not be completely excluded due to the possibility of overlapping proteins. Therefore, an approach to augment the plumbagin induction signal information and to offer an alternative method of confirming the responsiveness of the genes was explored. Theoretically, when a gene is cloned into a plasmid with its promoter, the responsiveness of the encoded proteins would be amplified due to the multiple-copy property of the plasmid. Therefore, the genes identified above together with a 5′ region, ranging from 425 bp to 1,100 bp upstream of the open reading frame, were PCR amplified. The obtained DNA fragments were separately cloned into the pMH vector, in which every single target protein is tagged with His_6 at the C terminus. Proteins displayed on the 1-D system could be simply compared by Western blotting with anti-His_6 tag antibody.

A bacterial transformant harboring plasmid pMH-mdaB treated with plumbagin was compared with those treated with DMSO alone. The MdaB protein increased about 2.5-fold (Fig. 3A) in the presence of plumbagin, given that the total proteins were loaded in comparable amounts (OmpC was used as an internal control; data not shown). Gene *mdaB* is known as "modulator of drug activity B" and has been recently proposed to function as an NADPH quinone reductase inducible by menadione (15, 19). Our results further demonstrated that *mdaB* also responded to plumbagin. The *nfo* gene encodes endonuclease IV and participates in the repair of DNA lesions induced by H_2O_2 (9). *nfo*'s response to plumbagin (Fig. 3B) is consistent with observations previously reported (3). An unknown protein encoded by *ygfZ* was identified as being upregulated for the first time (Fig. 3C). Expressions of AhpC protein (Fig. 3D), a peroxiredoxin (alkyl hydroperoxide reductase) offering antioxidant protection, and NfnB, an oxygen-insensitive

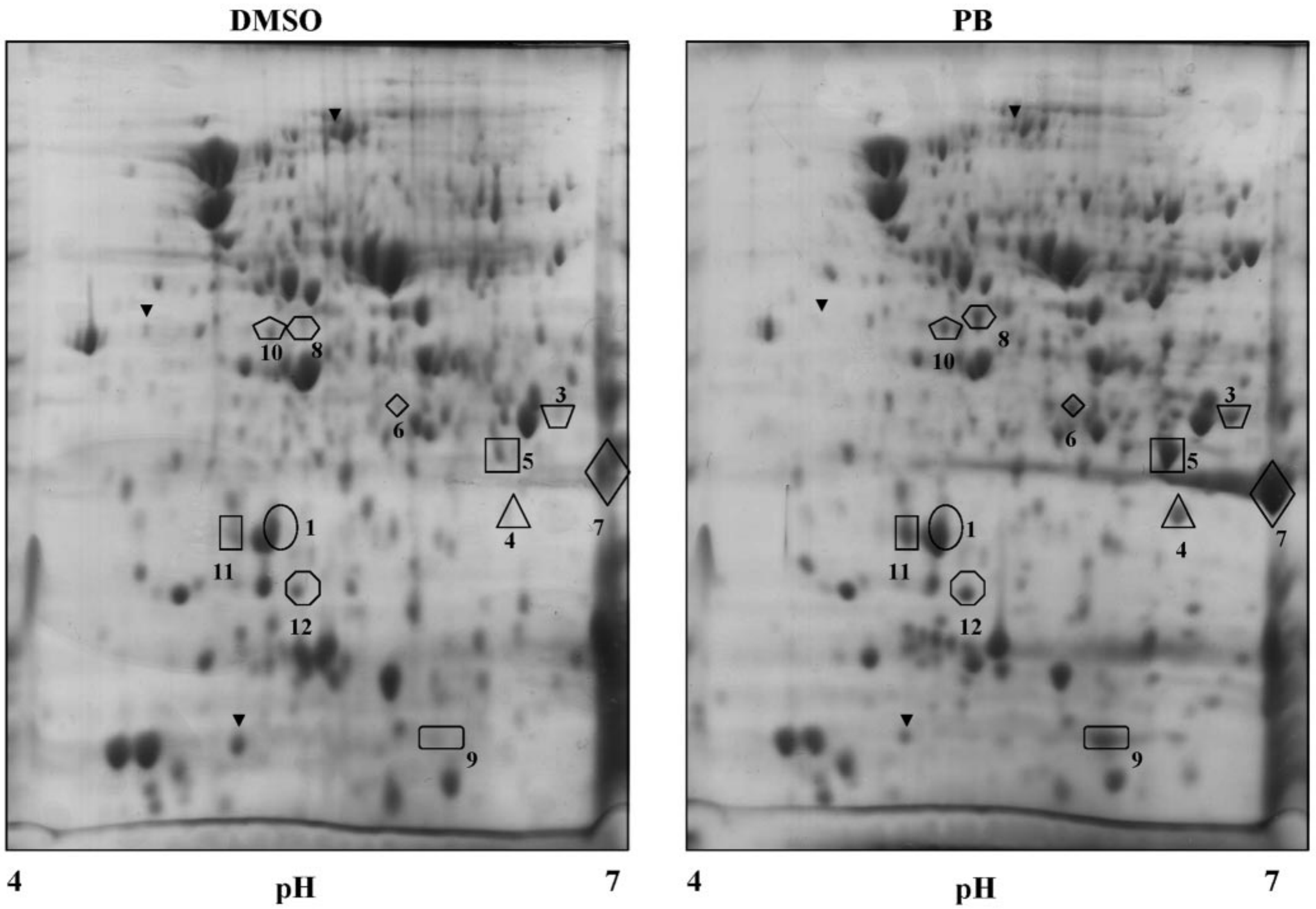

FIG. 1. Comparison of protein profiles of bacteria with and without plumbagin (PB) treatment. Protein samples were prepared in IEF buffer and separated by 1-D IEF with an Immobiline Dry strip at pI 4 to 7. This was followed by 2-DE. Thereafter, proteins on gels were stained with fresh Coomassie blue dye. The up-regulated protein spots highlighted were subject to Q-TOF mass spectroscopy identification. Proteins are numbered as shown in Fig. 2, and protein 2 ran off the gel and is not shown. The DMSO panel is the protein profile prepared from bacteria treated with DMSO only. It is worth noting that proteins up-regulated outnumbered those down-regulated. Representatives of the down-regulated protein spots are indicated by arrowheads.

NAD(P)H nitroreductase (Fig. 3E), were both confirmed to be up-regulated. Protein derived from *ahpC* was observed with an extra product which was twice as large as expected (Fig. 3D). This fact suggests that AhpC may form a dimer structure or associate with another molecule(s) when bacteria encounter oxidative stress. The induced protein pattern detected with NfnB was striking. At least five additional high-molecular-weight bands above the expected product were observed (Fig. 3E). These high-molecular-weight aggregates of NfnB have not been reported before. Multiple high-molecular-weight products were also seen when *gatY* was examined in the same manner in the presence of plumbagin (data not shown).

The positive responsiveness of the genes *fldA*, encoding flavodoxin I, and *yggX*, encoding a protein involved in Fe(II) trafficking to minimize DNA damage (13), was also confirmed in a similar way (data not shown). In contrast, three other genes listed in Fig. 2B, namely, *talB*, *tpx*, and *ygaG*, could not be confirmed and were therefore excluded from further investigation.

Marbox sequence contributed to plumbagin responsiveness. To explore the mechanism of genes' up-regulation upon plumbagin treatment, the 5′-flanking regions of these genes were compared. A stretch of sequence containing a putative marbox was observed in the regions upstream of *fldA*, *mdaB*, *nfnB*, *nfo*, *sodA*, and *yggX*. These sequences have been proposed to be the controlling elements, but so far only that of *yggX* has been characterized by binding of SoxS to a promoter-containing fragment and those of *mdaB* and *nfnB* have been mapped by fragment deletion in a promoter assay (28, 32). It is worth noting that those characterizations were not based upon the effects of plumbagin. Instead, they were based on observations with paraquat, 4,4′-dipyridyl, or salicylate.

To confirm the effects of plumbagin treatment on gene expression, a mutation was introduced into the mapped marbox sequence upstream of *mdaB* (28, 32). In the pMH-mdaB construct, the putative core element GCAC of the marbox (Fig. 4A) was replaced with GCGC, resulting in plasmid pMH-mdaBm. MdaB expression was then compared. The basal ex-

(A)

AhpC

MSLINTKIKPFKNQAFKNGEFIEITEKDTEGRWSVFFFYPADFTFVCPTELGDVADHYEELQK**LGV**
DVYAVSTDTHFTHKAWHSSSETIAKIK**YAMIGDPTGALTR**NFDNMREDEGLADR**ATFVVDPQG**
IIQAIEVTAEGIGRDASDLLRKIKAAQYVASHPGEVCPAKWKEGEATLAPSLDLVGKI

(B)

Number	Protein (residues)	Number of peptide sequence identified	Sequence coverage (%)	Annotation
1	AhpC (187)	3	28.3%	alkyl hydroperoxidase reductase, C22 subunit
2	FldA (176)	1	10.3%	flavodoxin 1
3	GatY (286)	3	9.1%	tagatose-bisphophate aldolase
4	MdaB (193)	3	30.6%	modulator of drug activity B
5	NfnB (217)	4	12.9%	oxygen-insensitive NAD(P)H nitroreductase
6	Nfo (285)	4	28.4%	endonuclease IV
7	SodA (206)	8	55.1%	superoxide dismutase
8	YgfZ (326)	4	15.3%	hypothetical protein
9	YggX (91)	4	53.8%	Yggx protein
10	TalB (317)	2	6.3%	transaldolase B
11	Tpx (168)	1	10.7%	thiol peroxidase
12	YgaG (171)	3	22.2%	AI-2 synthase

FIG. 2. Summary of proteins identified by Q-TOF mass spectroscopy after 2-DE comparison. (A) Complete amino acid sequence of AhpC with the identified peptides underlined. (B) Proteins deduced from Fig. 1 and listed in alphabetical order.

pression level of MdaB from pMH-mdaBm was fivefold decreased relative to that from pMH-mdaB (compare lanes 1 and 3 in Fig. 4B). Upon addition of plumbagin, the expression of MdaB from pMH-mdaB was 2.5-fold higher than when the solvent was added alone (lane 1 versus lane 2). In contrast, the increase in MdaB from the pMH-mdaBm transformant was limited to 1.3-fold when plumbagin was added (lanes 3 and 4). Therefore, a single base mutation of the marbox sequence drastically decreased the basal level of MdaB expression, as well as the plumbagin induction effect. In a similar approach, the proposed marbox of *nfo* (28, 32) was mutated (Fig. 4A) and the core-containing element CGCAT in pMH-nfo was mutated to TCTAG in pMH-nfom. The basal expression level of Nfo from pMH-nfom was lower than that derived from pMH-nfo (compare lanes 1 and 3 in Fig. 4C), and a 2.4-fold decrease was observed. On treatment with plumbagin, the induction effect of plumbagin was reduced from a 3.5-fold difference in the wild type to a 1.1-fold difference in the mutant (compare lanes 1 and 2 with lanes 3 and 4).

To examine the element conferring the plumbagin induction effects on the uncharacterized *ygfZ* gene, the sequence upstream of *ygfZ* was first analyzed with the Emboss Fuzznuc program (http://www.hgmp.mrc.ac.uk/Software/EMBOSS), and several marbox-like sequences were found in this region. To facilitate the identification of the plumbagin-responsive element, pMH-ygfZ was reconstructed to generate a shortened 5′-flanking region in pMH-ygfZ′ (Fig. 5A). Compared to pMH-ygfZ, the plumbagin-induced expression of *ygfZ* was retained in pMH-ygfZ′ (lanes 1 to 4, Fig. 5C). This result suggested that the plumbagin-responsive element exists in the 151-bp region upstream of the *ygfZ*-coding sequence. Analysis of this sequence in detail found a *zwf*-like class I marbox promoter structure (27, 36) that is characterized by a space of 7 bp between a putative marbox and a putative −35 hexamer. Therefore, the core-containing stretch, GCACA, in the putative marbox sequence was replaced with GTACC (see sequence alignment in Fig. 5B), and the expression levels of YgfZ before and after the sequence alteration were analyzed (Fig. 5C). The basal expression level of YgfZ from pMH-ygfZ′m was suppressed (compare lanes 3 and 5) by this two-base mutation compared to that from pMH-ygfZ′, and the plumbagin induction effect was abolished as well (compare lanes 5 and 6). The total effect is even obvious when lanes 4 and 6 are compared.

SoxS is involved in early plumbagin induction. Previous studies suggested that two marbox-binding proteins, SoxS and MarA, might be induced directly by treatment with plumbagin (2, 10). To differentiate their contributions, two plasmids containing their intrinsic control elements together with the appropriate *marA* and *soxS* genes were separately constructed. In pMH-marRA, the complete *marRA* locus was inserted into pMH and the product of *marA* was fused with a His_6 tag. The expressed MarA protein was then followed by Western blotting with anti-His_6 as in Fig. 3. Similarly, pMH-soxRS was constructed to detect the expression of SoxS. Bacteria transformed with pMH-marRA and pMH-soxRS, respectively, were treated with plumbagin, and the kinetics of MarA and SoxS expression were followed by Western blotting. Figure 6A shows that SoxS was robustly induced and strongly detected as early as 5 min after plumbagin treatment (Fig. 6A). In contrast, the induction

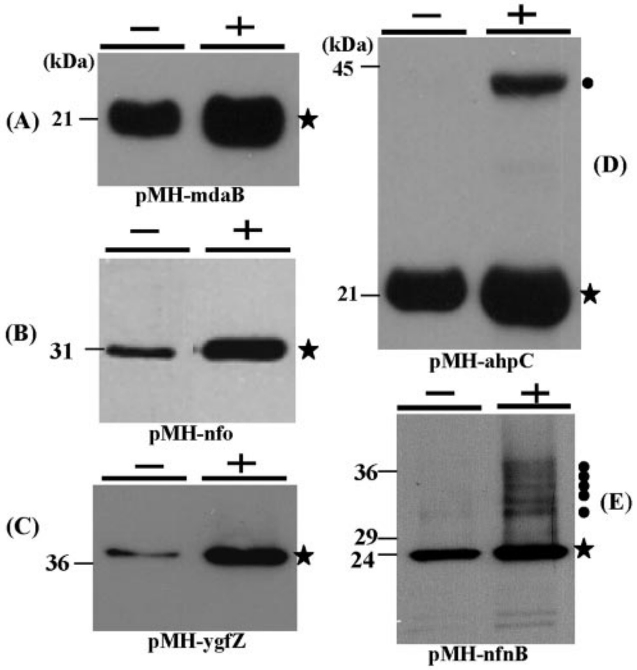

FIG. 3. Confirmation of the responsiveness of specific gene products induced by plumbagin. Bacteria transformed with the indicated plasmid and cultivated in the presence (+) or absence (−) of plumbagin (25 μg/ml). The total proteins separated by 1-D SDS-PAGE were analyzed by Western blotting for the expression of His$_6$-tagged protein. The target protein with the expected molecular size is indicated by an asterisk, whereas those with sizes greater than expected or equal to a putative dimer are indicated by dots. Comparable protein samples were loaded in each lane with OmpC, an outer membrane protein, as a reference (data not shown).

of MarA was negligible over the first 10 min and slowly increased thereafter. Figure 6B, with a graphic illustration, further sketches out the concept that SoxS is induced first in the early phase of the bacterial response to plumbagin. After 30 min, the level of SoxS reached a plateau while the level of MarR had a limited increase. With the MarA level induced by sodium salicylate (33) as a reference, the 60-min plumbagin treatment only reached about 60% of the level induced by salicylate. This slow MarA induction is unique to plumbagin treatment since the same bacteria gave a rapid plateau in response to sodium salicylate treatment for less than 30 min (Fig. 6C).

SoxS is involved in the plumbagin induction of some genes but not all. The robust induction of SoxS by plumbagin suggests a role for SoxS in the activation of these gene. Since our proteomic analysis was based upon bacteria after a 2-h induction, gene activation conferred by other late events may also be possible. To distinguish the genes controlled by SoxS from those controlled by other proteins, we constructed a *soxS* deletion mutant and repeated the experiments involving treatment with plumbagin. Figure 7A shows that a *soxS* deletion mutant gave a basal level of Nfo similar to that found with the parental strain (compare lanes 1 and 3). However, when encountering plumbagin, the *soxS* mutant totally lost the response (Fig. 7A, compare lanes 2 and 4). After complementation with a SoxS-expressing plasmid, pACYC-soxRS, the plumbagin responsiveness was completely restored (Fig. 7A, lanes 3 to 6). Therefore, plumbagin's effect on *nfo* was conceivably through a direct involvement of SoxS. Similar to *nfo*, *ygfZ* and *sodA* lost their reactions toward plumbagin when bacterial *soxS* was deleted (data not shown). Although not tested, *fldA* and *yggX* are presumably in the same category since they have been known to be regulated by SoxS (32, 37).

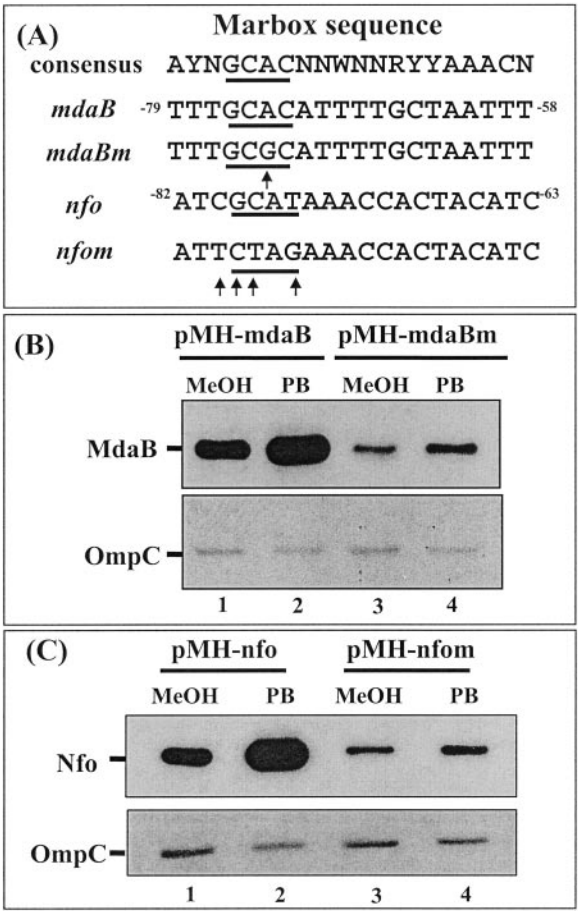

FIG. 4. Effects of marbox mutations on expression of plumbagin-responsive genes. (A) Alignment of the marbox sequences (26) with the most conserved GCAC core underlined. Base changes generated by mutagenesis are indicated by arrows. (B) Analysis of MdaB expression affected by the mutations shown in panel A. Plasmid-transformed bacteria were treated with plumbagin (PB) or with methanol (MeOH) alone. Total proteins of the bacteria were analyzed for expression of MdaB by Western blotting as in Fig. 3. Blots were also stained for OmpC, a sample loading control. (C) Analysis of the effects of marbox mutations on the expression of Nfo. Experiments were conducted similarly to those in panel B.

On the other hand, *mdaB*, *nfnB*, and *ahpC* substantially differed from *nfo*. Their products remained plumbagin inducible when *soxS* was deleted. A typical example with MdaB is shown in Fig. 7B.

DISCUSSION

The antibacterial effect of plumbagin against *E. coli* was observed under aerobic conditions. When plumbagin was present at one-half of the MIC, the bacteria continued to grow,

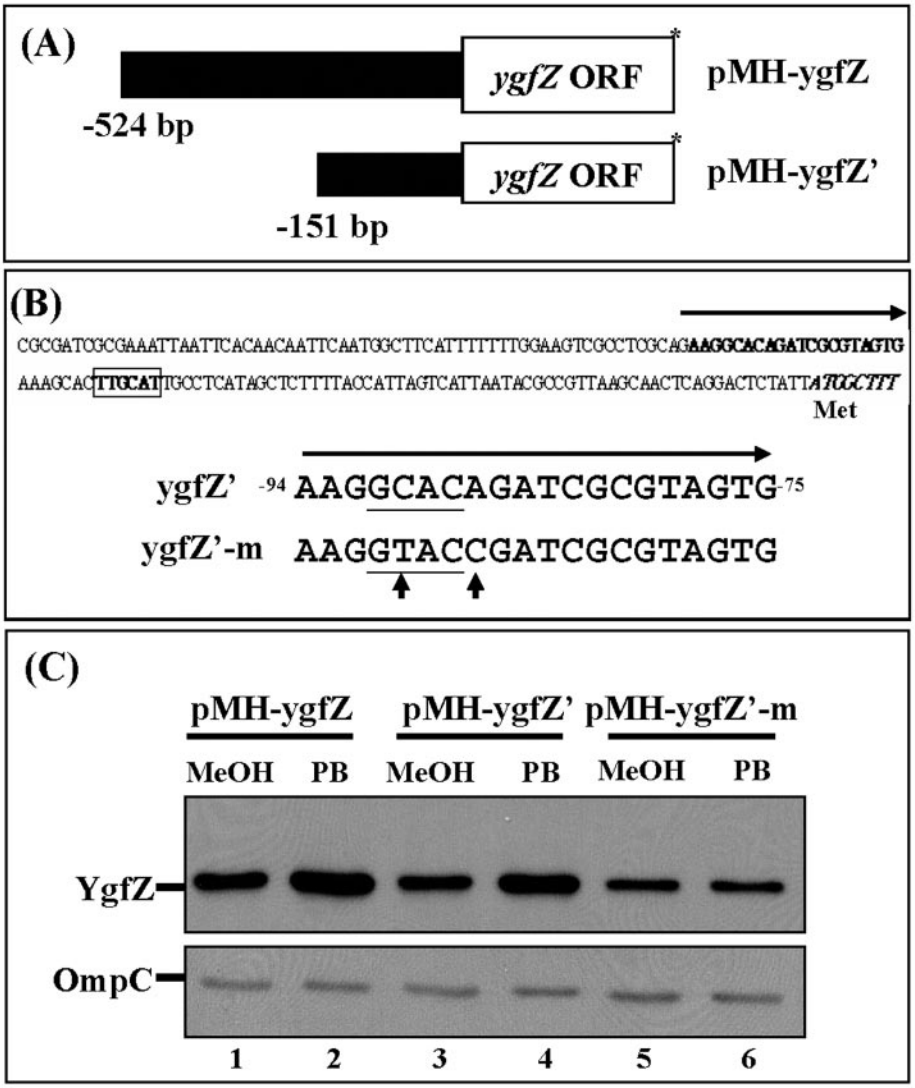

FIG. 5. Mapping of the putative marbox for *ygfZ* and monitoring of effects of mutations on gene expression. (A) Schematic of constructs containing the upstream sequence and the entire *ygfZ* coding frame. The filled box indicates the 5′-flanking region, the open box represents the open reading frame, and the asterisk indicates the C-terminal hexahistine tag. (B) Mutations created in the putative marbox located upstream of *ygfZ*. Vertical arrows indicate mutated bases. The nucleotide sequence upstream of the initiation codon of *ygfZ* is shown above the alignment; the putative marbox is indicated by the arrowed line, whereas the putative −35 region is boxed; and bases coding for the N terminus of YgfZ are in italics. (C) Comparison of the expression of *ygfZ* in three different constructs. Protein expression levels were monitored by Western blotting. Detection was carried out in a way similar to that used for Fig. 4. MeOH, methanol; PB, plumbagin.

but at a slow rate, and many gene products of *E. coli* were up-regulated. Twelve proteins significantly elevated have been identified by proteomic analysis, and nine of the genes involved have been reconfirmed by an alternative approach. Intriguingly, more than one-half of plumbagin-responding genes could be functionally correlated with antioxidation, physiological reduction, and DNA lesion repair (1, 8, 11, 23, 24, 32, 34). Considering that plumbagin has no obvious effect on *E. coli* growth in the absence of oxygen and that there was no recognizable difference between the 2-DE protein profiles of *E. coli* cells treated with and without plumbagin (data not shown), the toxic effect of plumbagin on *E. coli* must be reconsidered.

The elevated expression of the genes *fldA*, *mdaB*, *nfnB*, *nfo*, *sodA*, and *yggX* caused by plumbagin in our study was also observed in a microarray study when bacteria were treated with paraquat (31). These genes have been defined as members of the mar/sox regulon, and their regulation has been proposed to occur through marbox sequences that share conserved cores with otherwise degenerated sequences (28). We have mutated the marbox sequences in front of *mdaB* and *nfo* and proved that such mutations indeed decreased the basal level of the proteins expressed, as well as their responses to plumbagin. We rationalize that *fldA*, *nfnB*, *sodA*, and *yggX* may be regulated by similar *cis* elements. As to *trans* factors, the plumbagin induction effects on *nfo*, *fldA*, *sodA*, and *yggX* were found to be exclusively dependent upon SoxS. Additional regulation at the posttranscriptional level could not be completely excluded. On the other hand, the elevated MdaB and NfnB protein levels

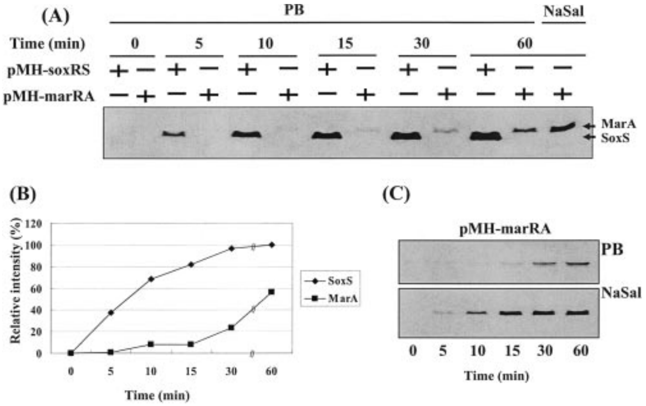

FIG. 6. Kinetics of MarA and SoxS induction in *E. coli*. (A) Western blotting analysis of MarA and SoxS induction in bacteria harboring different plasmids and treated with the chemicals indicated. Bacteria were harvested at different time intervals, and the total proteins dissolved in the SDS sample buffer were subjected to 1-D SDS-PAGE and analyzed by Western blotting with anti-His tag antibody. Protein loading was controlled with OmpC as a reference (data not shown). (B) Quantification of images shown in panel A by densitometry. The band intensity of SoxS after bacteria were treated with plumbagin (PB) for 60 min and that of MarA after similar treatment with sodium salicylate (NaSal) at a final concentration of 5 mM were set as references, respectively. (C) Comparison of MarA kinetics induced by plumbagin versus sodium salicylate. Experiments were carried out similarly to that in panel A.

caused by plumbagin were apparently not affected regardless of the presence or absence of *soxS*.

In our study, *gatY* was also up-regulated, and this induction has not been observed with paraquat (28). Since the expression of genes involved in sugar transport has been elevated when *E. coli* encounters superoxide stress (28, 31) and since *gatY* has been known to encode a protein involved in carbohydrate utilization, it is not surprising to see the up-regulation of *gatY* by plumbagin. However, no marbox-like sequence was found proximal to *gatY* previously (28) and we were also unable to identify a related sequence. Therefore, how this gene is molecularly regulated remains to be explored.

A gene whose function is unknown that was identified in our plumbagin treatment is *ygfZ*; the crystal structure of the protein it encodes has been reported, and a folate-dependent regulatory role in one-carbon metabolism has been hypothesized. However, its physiological role and real function remain unclear (35). We found that the *ygfZ* gene contains a *zwf*-like class I marbox structure, and mutation of this sequence did decrease the basal protein level, as well as its response to plumbagin, a reaction that was also *soxS* dependent. Therefore, these new findings suggest that *ygfZ* may also be involved in releasing oxidative stress.

ahpC has been reported to be regulated by OxyR (34), and it is also stimulated by plumbagin. This fact strongly suggests that plumbagin not only generates superoxide stress but also provokes the production of peroxide ions to trigger a mixed set of responses in *E. coli*. In our *soxS* deletion mutant, the plumbagin effect with an increasing AhpC level remained evident, a fact suggesting that SoxS does not act as a mediator. It is then suggested that the readily induced MarA protein, albeit at a slow response, and Rob (18), which is known to bind to sites with similar sequences and was not monitored here, may contribute to the increase in AhpC. The same reasoning may be valid with the findings on MdaB and NfnB, on neither of which the plumbagin effect was SoxS dependent. Whether the above conclusions found with *E. coli* are applicable to bacteria that are sensitive to plumbagin under both aerobic and anaerobic conditions remains to be explored.

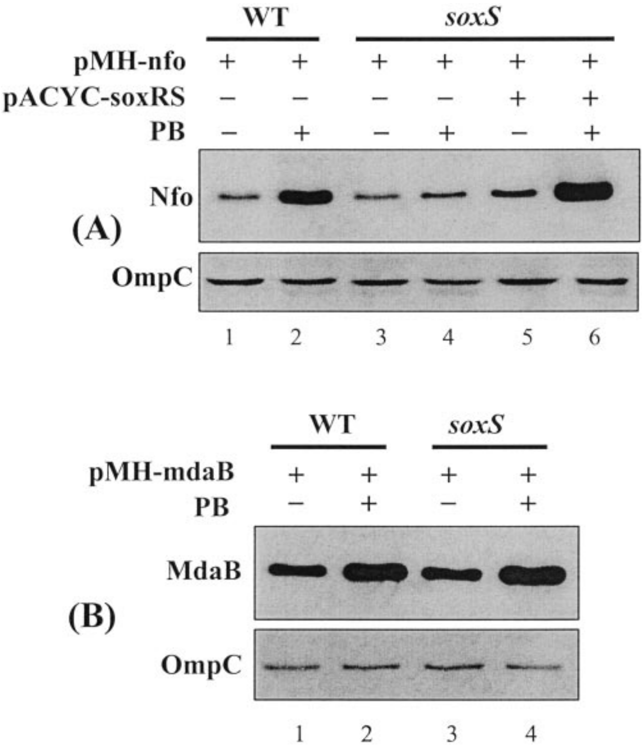

FIG. 7. Analysis of the plumbagin induction effect in a *soxS* deletion mutant. (A) Increasing detection of Nfo in bacteria treated with plumbagin is *soxS* dependent. *E. coli* parental strain 110 (WT) and its *soxS* deletion mutant (*soxS*) were transformed with pMH-nfo, treated with plumbagin, and analyzed for expression of Nfo as described in the legend to Fig. 3. The lost SoxS in the deletion mutant was complemented by expression from pACYC-soxRS, which has a plasmid *ori* compatible with that of pMH-nfo. (B) The presence or absence of *soxS* has no apparent effect on the plumbagin-induced increase in MdaB.

Under our plumbagin treatment conditions, bacterial proteins whose levels were increased appeared to outnumber those being suppressed (Fig. 1). These studies of up-regulated proteins do not necessarily imply that those that are down-regulated are not important for bacterial survival. As proteomic investigations increase both protein resolution and staining sensitivity, it is expected that more protein spots on either the up-regulated or the down-regulated side will be unveiled. By grouping responsive genes and gene products, how bacteria use different mechanisms to resolve the same chemical stress may be better understood.

ACKNOWLEDGMENTS

We thank J. C. W. Lio for the gift of pMH, J. Y. Ho for operating the mass spectrometer, and C. M. Tzeng of U-Vision Biotech for continuous encouragement. We also thank R. Kirby for critically reading the manuscript.

This research was supported in part by grant 89-B-FA22-2-4 (Program for Promoting Academic Excellence of Universities) from the Ministry of Education and grant NSC 94-2320-B-010-034 from the National Science Council, Taiwan, Republic of China.

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