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SoxRS-Mediated Lipopolysaccharide Modification Enhances Resistance against Multiple Drugs in *Escherichia coli*[▽]

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Lipopolysaccharide (LPS) is a major constituent of the outer membrane of gram-negative bacteria that serves as a barrier against harmful molecules, including antibiotics. The *waaYZ* locus that encodes the LPS core biosynthetic function in *Escherichia coli* was found to be induced strongly by superoxide generators but not by H_2O_2, ethanol, or heat shock. This induction was dependent on SoxRS, a superoxide and nitric oxide sensing system, through a soxbox in the *waaY* promoter that binds SoxS. A Δ*waaYZ* mutant became more sensitive to some superoxide generators, and the activation of SoxR by these drugs became more sensitized in the mutant. Through phenotypic microarray analysis, we found that the mutant became sensitive to a wide variety of chemicals not restricted to oxidizing agents. We found that the mutant is under envelope stress and is altered in LPS composition, as monitored by the level of σ^E activation and changes in the electrophoretic mobility of LPS, respectively. *waaY* expression was also regulated by MarA (multiple-antibiotic resistance regulator), which shares a binding site (soxbox) with SoxS, and was induced by salicylate, a nonoxidative compound. These results demonstrate a novel way of protecting gram-negative bacteria against various compounds by modifying LPS, possibly through phosphorylation. Since either oxidant or nonoxidant compounds elicit resistance toward themselves and other toxic drugs, this mechanism could serve as an efficient way for pathogenic bacteria to enhance survival during antibiotic treatment within an oxidant-rich host immune environment.

Living organisms have evolved efficient mechanisms to sense environmental stresses and to control the expression of related defense genes. Bacterial defense mechanisms against oxidative stress and antibiotic drugs are of particular interest because both are used by pathogenic bacteria to survive the phagocytic attack of immune cells that generate reactive oxygen species (ROS) and to escape from antibiotic medication. Antibiotic resistance in bacteria often arises from the acquisition of antibiotic-specific resistance genes or from a broader mechanism against multiple antibiotics. In *Escherichia coli*, the *mar* regulon (*m*ultiple *a*ntibiotic *r*esistant) confers resistance not only to multiple antibiotics but also to organic solvents and disinfectants (2). MarR, a repressor of the *marR-marAB* operon, is inactivated by some antibiotics and phenolic compounds to derepress *marR-marAB* expression. MarA activates the expression of diverse genes, including *acrAB*, *micF*, *mlr-1*, *-2*, and *-3*, *slp*, and *inaA*, which endow cells with resistance (3).

The response to oxidative stress is mediated through two major regulatory systems in *E. coli*, namely, OxyR, targeted toward peroxides, and SoxRS, targeted toward superoxide and nitric oxides (27). Both contribute to increased survival of *E. coli* against oxidative attack by the host immune system. SoxR serves as a sensor for superoxide and nitric oxide through its [2Fe-2S] center and activates *soxS* transcription when oxidized. The increased level of SoxS then activates the expression of target genes that repair damaged DNAs, maintain the redox balance, and defend against toxic radicals.

The close relationship between the oxidative stress response and antibiotic resistance is manifested in the extensive overlap between *soxRS* and the *mar* regulon (7, 26) and was highlighted by a recent report that the killing mechanism of bactericidal antibiotics involves oxidative damage (16). The *mar* regulon includes many genes that are regulated by SoxRS in response to oxidative and nitrosative stresses (3, 21). This is due to the close relatedness of the two regulators SoxS and MarA (21), which bind to a common set of promoters with a regulatory sequence called either the "soxbox," for SoxS binding, or the "marbox," for MarA binding (3, 21). Although these promoters are not stimulated to the same extent by both activators, the members of the *soxRS* and *mar* regulons can roughly be regarded as the same.

More than 60 direct target genes of SoxRS and MarA have been catalogued, with functions related to drug resistance (*acrAB*, *tolC*, *marAB*, and *micF*), iron homeostasis (*fur*, *yggX*, and *fpr*), reducing oxidants (*sodA* and *zwf*), DNA repair (*nfo*), oxidant-resistant isoenzymes (*fumC* and *acnA*), and others (*ribA* and *pqi-5*) (7, 14, 15, 26, 27). Except for those related to drug efflux and outer membrane porin regulation, most genes are related to intracellular functions. In this study, we present a new target gene of the SoxRS system that modifies lipopolysaccharide (LPS) in the cell envelope and provides resistance against a broad range of drugs that include oxidants and antibiotics. This provides a new example of a mechanism for the cross talk between the oxidative stress response and drug resistance, which will enable pathogenic bacteria to survive the oxidative host defense and antibiotic medications.

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TABLE 1. Bacterial strains, plasmids, and phages used in this study

Strain, plasmid, or phage	Description[a]	Reference or source
Strains		
GC4468	(*argF-lac*)*169 rpsL sup*(Am)	35
DH5α	F^- ϕ80d*lacZ*ΔM15Δ(*lacZYA-argF*)*U169 endA1 recA1 hsdR17 deoR gyrA96 thi-1 relA1 supE44*	28
BW829	GC4468 *sox-8*::*cat*	36
BW831	GC4468 *soxS3*::Tn*10*	36
BW847	GC4468 *soxR4*::*cat*	36
BW900	GC4468 *soxR9*::*cat*	38
GSO18	*recD1014 lac gal rpsL* Δ*oxyR*::*kan*	4
JWK5249	W3110 Δ*marA*::*kan*	6
JH101	GC4468 Φλ*waaYp* (−101 to −4; H73)-*lacZ lacY*$^+$ *bla*$^+$	This work
JH201	JH101 *sox-8*::*cat* P1(BW829)	This work
JH301	JH101 *soxS3*::Tn*10* P1(BW831)	This work
JH401	JH101 *soxR4*::*cat* P1(BW847)	This work
JH501	JH101 *oxyR*::*kan* P1(GSO18)	This work
JH103	GC4468 Φλ*waaYp* (−53 to +96)-*lacZ lacY*$^+$ *bla*$^+$	This work
JH104	GC4468 Φλ*waaYp* (−35 to +96)-*lacZ lacY*$^+$ *bla*$^+$	This work
JH203	JH103 *sox-8*::*cat* P1(BW829)	This work
JH603	JH103 P1(JWK5249)	This work
MS1343	GC4468 Φλ*soxS′-lacZ*	17
BM900	MS1343 *soxR9*::*cat*	17
CAG16037	MC1061 Φλ*rpoHP3-lacZ*	11
CP367	*polA* (Ts)	Lab collection
JH1001	GC4468 Δ*waaY*::*kan*	This work
JH1003	GC4468 Δ*waaYZ*::*kan*	This work
JWK3600	W3110 Δ*waaY*::*kan*	6
Δ$waaY_{inf}$	GC4468 with in-frame deletion of *waaY*	This work
JH2003	MS1343 Δ*waaYZ*::*kan*	This work
JH2004	BM900 Δ*waaYZ*::*kan*	This work
JH3003	CAG16037 Δ*waaYZ*::*kan* P1(JH1003)	This work
Plasmids		
pRS415	*lacZYA* operon fusion vector; Amp^r	32
pJH97	97 bp of *waaYp* (−101 to −4; H73) cloned into pRS415; Amp^r	This work
pJH98	149 bp of *waaYp* (−53 to +96) cloned into pRS415; Amp^r	This work
pJH99	131 bp of *waaYp* (−35 to +96) cloned into pRS415; Amp^r	This work
pTac3N	Protein expression vector under *tac* control; Apr^r	This work
pWaaY	WaaY expression plasmid (*waaY* open reading frame cloned into pTac3N)	This work
pWaaYZ	WaaYZ expression plasmid (*waaYZ* open reading frame cloned into pTac3N)	This work
Phages		
λRZ5	(*bla′-′lacZ*) *lacY*$^+$	Lab collection
$P1_{vir}$	Virulent derivative of P1 phage	31

[a] Amp, ampicillin; Kan, kanamycin; Apr, apramycin.

MATERIALS AND METHODS

Strains, phages, and plasmids. All strains, phages, and plasmids used in this study are listed in Table 1. DH5α was used for the cloning of recombinant DNA, and GC4468 was used as a host strain to harbor chromosomal copies of various *lacZ* fusions and mutations. The promoter-probing plasmid pRS415, which contains the promoterless *lacZYA* genes, was used for the construction of promoter-*lacZ* fusions.

Culture conditions. LB medium (1% tryptone, 1% NaCl, and 0.5% yeast extract) was used for routine bacterial culture. Antibiotics were used at the following concentrations: ampicillin, 50 μg/ml; kanamycin, 25 μg/ml; tetracycline, 20 μg/ml; and chloramphenicol, 20 μg/ml. To determine the effects of various chemicals on gene expression, the *lacZ* fusion strains were grown in LB broth to an optical density at 600 nm (OD_{600}) of 0.2 with vigorous shaking, treated with the agents at the indicated concentrations for 1 h, and assayed for β-galactosidase activity as described by Miller (22).

DNA and RNA manipulation. Reactions for DNA manipulation were carried out according to standard protocols or as recommended by the manufacturers. We always confirmed the final sequences of the constructs after every recombination process with DNA. Cellular RNA was extracted with Ultraspec-II total RNA isolation kits (Biotecx Laboratories Inc.) as recommended by the manufacturer, except that the cells were first treated with lysozyme (4 mg/ml) in 50 mM glucose, 25 mM Tris-HCl (pH 8.0), and 10 mM EDTA for 5 min on ice.

Construction of single-copy *lacZ* fusions and $P1_{vir}$ transduction. *waaY* promoters of various lengths were cloned into pRS415 (pJH97, pJH98, and pJH99) and transformed into GC4468. The resulting transformants were infected with phage λRZ5 to bring about homologous recombination between the plasmid and phage DNAs in vivo, as described previously (32). The recombinant phage were then lysogenized into GC4468 at the *att* site to make single-copy lysogens (JH101, JH103, and JH104), which were screened by the lowest basal level of β-galactosidase activity. Introduction of various mutations into these *lacZ* fusion strains was done through $P1_{vir}$ transduction as previously described (31). The *soxRS*, *oxyR*, and *waaYZ* mutations were transduced from BW829 (*sox-8*::*cat*), BW831 (*soxS3*::Tn*10*), BW847 (*soxR4*::*cat*), BW900 (*soxR9*::*cat*), GSO18 (Δ*oxyR*::*kan*), JWK5249 (Δ*marA*::*kan*), JH1001 (Δ*waaY*::*kan*), and JH1003 (Δ*waaYZ*::*kan*) into the recipient strains, such as GC4468 and *soxS-lacZ* (MS1343), *rpoH* P3-*lacZ* (CAG16037), and *waaY-lacZ* (JH101 and JH103) mutants.

Primer extension and Northern hybridization. Primer extension and Northern analysis were done as described by Sambrook et al. (28). For primer extension, an oligonucleotide (5′-AATAATTGATTTCGCATCTCGTGG-3′) (see Fig. 2B) complementary to the downstream region of the putative +1 site was labeled at the 5′ end with [γ-^{32}P]ATP (Amersham) by T4 polynucleotide kinase. One hundred micrograms of RNA and 5′-end-labeled primer (10^4 to 10^5 cpm) were hybridized, and cDNA was synthesized by avian myeloblastosis virus reverse

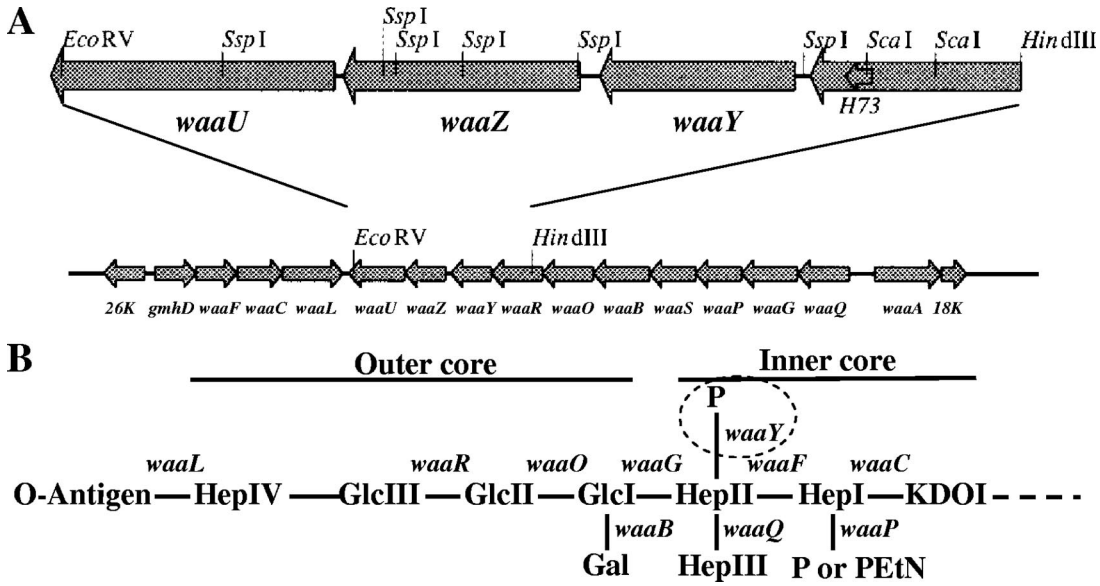

FIG. 1. *waa* gene cluster of *E. coli* K-12, location of the paraquat-inducible promoter, and structure of the LPS core region and action site of WaaY. (A) *waa* gene cluster and *waaYZU* region. The small arrow in *waaR* indicates the location of the H73 promoter. (B) Structure of the LPS core region of *E. coli* K-12 and genes involved in biosynthesis, shown at their approximate sites of action. P, phosphate; Hep, heptose; Glc, glucose; Gal, galactose; KDO, 3-deoxy-D-manno-2-octulosonic acid; PEtN, 2-aminoethyl phosphate. The proposed site of *waaY* action is circled.

transcriptase (Promega). The resulting cDNAs were analyzed by electrophoresis on a 6% polyacrylamide gel containing 7 M urea. For Northern analysis, denatured RNA samples were electrophoresed in a 1% agarose gel containing formaldehyde and transferred to a Hybond-N+ membrane (Amersham). The 809-bp SspI-SspI fragment covering the whole *waaY* gene and upstream 303-bp HindIII-ScaI fragment (Fig. 1A) were used as probes that were labeled by the random priming method.

Gel mobility shift assay. The 97-bp AluI-AluI fragment of the *waaY* promoter (Fig. 2B) was size fractionated from an agarose gel and labeled with [α-^{32}P]dATP, using Klenow DNA polymerase. DNA binding reaction mixtures (20 μl) contained 10 mM Tris-HCl (pH 8.0), 75 mM KCl, 2 mM dithiothreitol, 10% (vol/vol) glycerol, 1 fmol of ^{32}P-labeled probe, 10 ng of poly(dI-dC):poly(dI-dC) (Sigma), and the indicated amounts of purified SoxS. The reaction mixture was electrophoresed in a 5% polyacrylamide gel (20 mM Tris-HCl, pH 8.0, 3 mM sodium acetate, 1 mM EDTA) and visualized by autoradiography on X-ray film or by phosphorimaging. HaeIII-digested fragments of pGEM-3zf(+) (Promega) and unlabeled probe were used as nonspecific and specific competitors, respectively. The SoxS protein used in this experiment was a native form which was overexpressed and purified as described by Li and Demple, using the SoxS expression plasmid pKOXS (20).

Gene disruption. Disruption of the *waaY*, *waaZ*, and *waaYZ* genes was done as described by Nagano et al. (25). The internal regions of the *waaY*, *waaZ*, and *waaYZ* genes (SspI-BssHII, ApaI-NsiI, and SspI-NsiI fragments, respectively) were displaced in vitro with a kanamycin/bleomycin resistance cassette from pUC4-KIXX (Pharmacia). The recombinant plasmids were transformed into a temperature-sensitive *polA* mutant (CP367) in which only cointegrates can form colonies at 42°C on antibiotic-containing plates. The cointegrates isolated at 42°C were further grown without antibiotics at 30°C for five consecutive generations to allow excision of the plasmid body by a second recombination event. The desired mutations were selected from Kan^r Amp^s colonies and transferred from the CP367 to GC4468 background by P1$_{vir}$ transduction. The correct gene replacement in all mutants was confirmed by Southern hybridization. In-frame deletion of *waaY* was done as described by Baba et al. (6). The *waaY*::*kan* locus was transferred from JWK3600 to GC4468 by P1$_{vir}$ transduction. The kanamycin resistance cassette was excised from the flanking FRT site by using an FLP helper plasmid (pCP20; Amp^r Chl^r), which was then removed by cultivation without antibiotics at 37°C. Colonies that lost all resistance were selected by replica plating. The correct in-frame deletion of the *waaY* gene was confirmed by PCR and sequencing.

LPS extraction and electrophoresis. LPS was extracted from whole cells by the hot phenol method as described by Chart (10). An overnight culture of cells was harvested, washed, and resuspended in TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA). An equal volume of hot phenol (68°C) was added, and the culture was mixed carefully to form a uniform "milky" emulsion and incubated at 68°C for 15 min. After centrifugation at 3,000 × *g* for 45 min, the upper, aqueous phase was collected. Further extraction by cold phenol was done twice to remove remaining proteins and lipids. LPS in the aqueous pool was electrophoresed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by silver staining.

PM. Phenotypic microarray (PM) analysis was performed by Biolog Inc., essentially as described elsewhere (9). Briefly, this analysis uses a redox chemistry employing cell respiration as a reporter. If the treatment of a drug is strongly positive for cell growth, the cells respire actively, reducing a tetrazolium dye and forming a strong color. If it is weakly positive or negative, the respiration is slowed or stopped, and less color or no color is formed. The differences in colorimetric intensities of the wild-type and mutant cells were recorded, with the colorimetric scores for the wild-type cells set as a reference. Thus, positive differences mean that the drug treatment enhanced mutant cell growth, and negative scores mean that the mutant cells became sensitive to the drug. In our study, the *waaYZ* mutant (JH1003) was compared pairwise with the isogenic parental strain (GC4468). The consensus results were taken from two independent runs with each of the two strains.

RESULTS

Oxidant-responsive promoter of *waaY*. Among paraquat-inducible promoters that were screened through cloning with the promoter-probing plasmid pRS415 (18, 19), a strongly induced one (H73) was located upstream of the *waaY* (formerly *rfaY*) gene, in the middle of the LPS core biosynthetic gene cluster (Fig. 1A). The *waaQGPSBORYZU* cluster has been suggested to constitute a single transcription unit, based on genetic studies (13, 29), and the WaaY protein has been suggested to be a kinase that phosphorylates HepII, a heptose of the LPS inner core (Fig. 1B) (40). The syntenic organization of the *waaY* gene is conserved in most *E. coli* strains, *Shigella* spp., and *Salmonella* spp. (13).

To precisely locate the inducible transcription start site of the *waaY* promoter (*waaYp*), we performed primer extension analysis of *waaY* transcripts in vivo. The results showed a

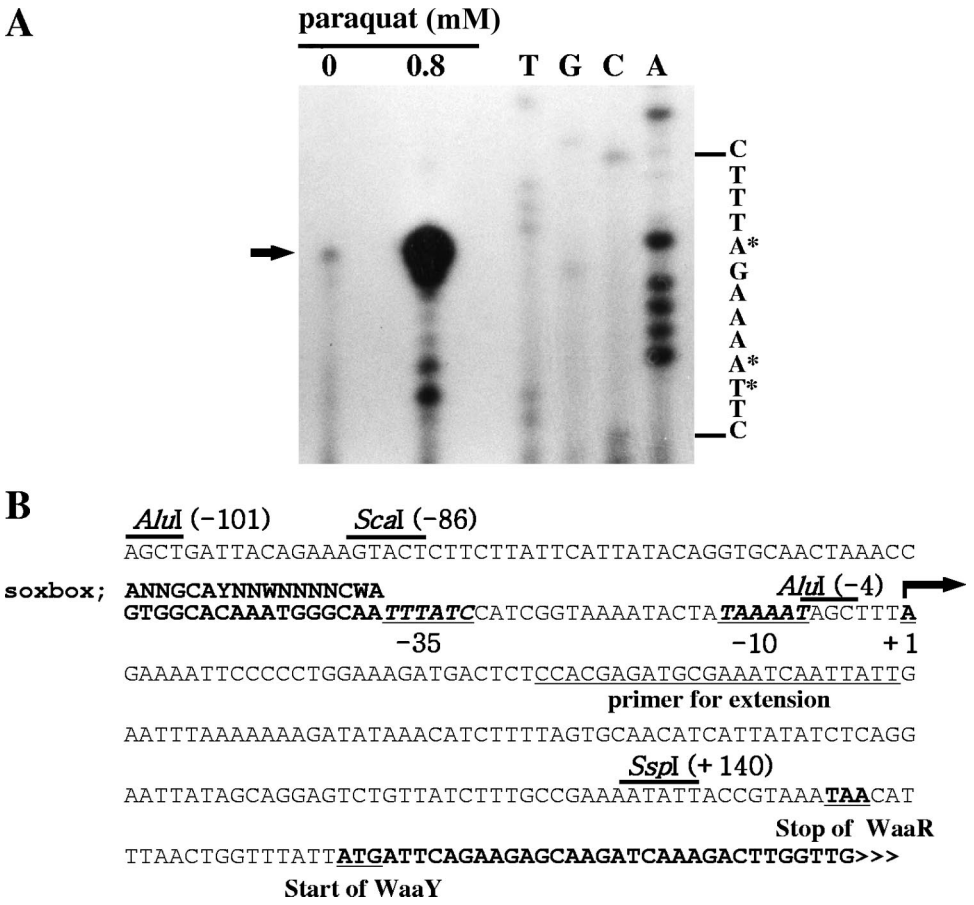

FIG. 2. Primer extension analysis and sequence of *waaYp* region. (A) Primer extension was carried out with total RNAs from paraquat-treated (0.8 mM) and untreated wild-type cells. +1 sites are indicated by arrows and asterisks. Growth conditions for RNA extraction were the same as those for the β-galactosidase assay. Induction was quantified as 19-fold by phosphorimager analysis (Bio-Rad). (B) Sequence of the *waaY* promoter region and details.

dramatic induction of the transcripts by paraquat, and the primary start site was mapped 171 nucleotides upstream from the start codon for WaaY (Fig. 2A). Minor RNAs of shorter lengths might reflect either minor downstream start sites or non-full-length extension. From the major transcriptional start site (+1), the −35 and −10 elements of the paraquat-responsive *waaYp* were predicted. A consensus sequence for SoxS binding (soxbox) was located adjacent to the −35 element (Fig. 2B). The inducibility of *waaYp* by various oxidants was examined by monitoring β-galactosidase activity from a *waaYp-lacZ* fusion integrated into the chromosome as a single copy (JH101 strain). As demonstrated in Fig. 3, *waaYp* responded dramatically to superoxide generators, such as paraquat, lawsone, menadione, and plumbagin, and much less to H_2O_2 and ethanol. It did not respond to heat (42 to 50°C) or a reducing agent (dithiothreiol) (data not shown).

SoxRS-dependent regulation of *waaY* transcription. Since *waaYp* responded almost exclusively to superoxide generators, we examined whether it is controlled by SoxRS. For this purpose, we introduced various *soxRS* and *oxyR* mutant loci into JH101 and estimated the expression of *waaYp-lacZ* (Fig. 4). As expected, the *soxRS* mutations (*sox-8* and *soxS3*) abolished the paraquat induction of *waaYp*, whereas a *soxR* constitutive mu-

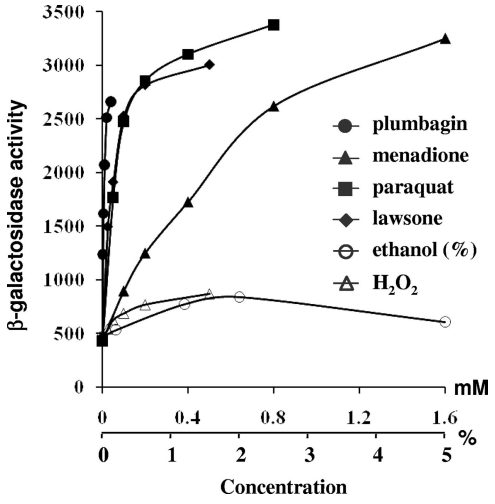

FIG. 3. Response of *waaYp* to various chemicals. Single-copy *waaYp-lacZ* fusion cells (JH101) in early exponential phase (OD_{600} = 0.2) were treated aerobically with various concentrations of chemicals for 1 h at 37°C and then assayed for β-galactosidase activity (Miller units). The concentration of ethanol is indicated separately (%). These are the most representative results from multiple measurements.

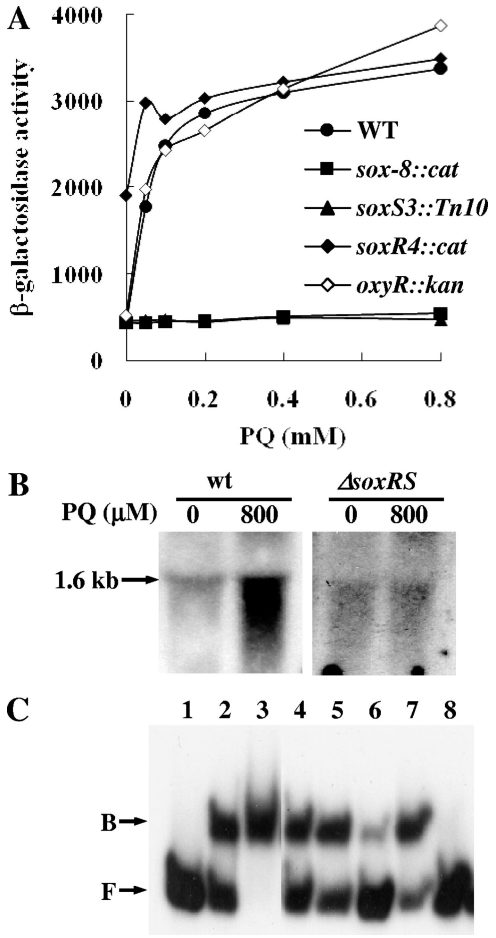

FIG. 4. *soxRS*-dependent induction of *waaY*. (A) *waaYp* activity was assayed in various *soxRS* and *oxyR* mutant backgrounds, using single-copy *lacZ* fusions. The isogenic wild type (WT) is JH101, and all mutants are derivatives of JH101: the *sox-8*::*cat* mutant (JH201) has a deletion mutation of *soxRS*, the *soxS3*::Tn*10* mutant (JH301) has an insertion mutation of *soxS*, the *soxR4*::*cat* mutant (JH401) has a constitutive *soxR* mutation, and the *oxyR*::*kan* mutant (JH501) has an insertion mutation of *oxyR*. β-Galactosidase activity was assayed after paraquat (PQ) treatment for 1 h with vigorous aeration. (B) Northern analysis was done with RNAs from the wild type (GC4468) and the *soxRS* mutant (BW829). An SspI-SspI fragment including the entire *waaY* open reading frame was used as a probe (Fig. 1A). A 1.6-kb transcript was induced 11.3-fold (quantified by phosphorimaging). (C) A 98-bp AluI-AluI fragment, from positions −101 to −4 (Fig. 2B), was used as a probe for a gel shift assay with increasing amounts of purified SoxS. Lanes 1 to 3, 0, 65, and 130 ng of purified SoxS, respectively (0, 250, and 500 nM, respectively); lanes 4 to 8, 130 ng of SoxS (500 nM), with nonspecific competitor in 65-, 130-, and 650-fold molar excess (lanes 4 to 6, respectively) or with specific competitor in 5- and 10-fold molar excess (lanes 7 and 8, respectively) over the labeled probe.

tation (*soxR4*) elevated the expression in the absence of paraquat. The *oxyR* mutation did not affect induction. Therefore, it is clear that *waaYp* is under the control of SoxRS. To estimate the size of the *waaY* transcript, we performed Northern analysis (Fig. 4B). The size of the induced RNA, which was observed in the wild type but not in the *soxRS* mutant, was about 1.6 kb. This is much larger than the length of the *waaY* coding region and is predicted to encompass most of the *waaZ* gene. Since a probe upstream of *waaYp* (303-bp HindIII-ScaI fragment) (Fig. 1A) failed to detect paraquat-inducible transcripts (data not shown), *waaYp* most likely produces a *waaY* (or, at maximum, *waaYZ*) transcript under conditions of superoxide stress.

We then examined the direct binding of purified SoxS protein to the DNA fragment containing the putative soxbox and found that it binds specifically to the *waaYp* fragment (Fig. 4C). An in vitro transcription assay also demonstrated that SoxS acts as a sole activator for RNA polymerase containing σ^{70} to transcribe *waaYp* (data not shown).

***waaYZ* mutants become sensitive to menadione and plumbagin but not to paraquat.** To find out the role of *waaYZ* genes in the oxidative stress response, we constructed Δ*waaY*, Δ*waaZ*, and Δ*waaYZ* mutants and investigated their sensitivity to superoxide generators. While both Δ*waaY* and Δ*waaYZ* mutants became sensitive to menadione and plumbagin, the Δ*waaY* mutant was a bit less sensitive than the Δ*waaYZ* mutant, and the Δ*waaZ* mutant was only slightly sensitive to these agents (data not shown). This indicated that *waaY* might play a major role in protection, but there might be a minor involvement of downstream genes, either *waaZ* or possibly *waaU*. To better address this possibility, we constructed an in-frame deletion mutant of *waaY* (Δ$waaY_{inf}$) to exclude polar effects and performed a complementation test with a plasmid encoding WaaY or WaaYZ. Similar results were obtained, showing that the Δ$waaY_{inf}$ mutant had significant sensitivity to menadione and plumbagin, but to a lesser extent than that of the Δ*waaYZ* mutant (Fig. 5A). This phenotype was complemented by the plasmid carrying *waaY*, confirming the major role of WaaY, but the Δ$waaY_{inf}$ mutant sometimes showed partial complementation (Fig. 5A). We suggest that *waaY* plays an important role in resistance, but we do not rule out the contribution of downstream *waaZ* for full activity.

Unexpectedly, *waaYZ* mutations did not increase the sensitivity to paraquat, and the Δ$waaY_{inf}$ mutant was even slightly resistant to paraquat (Fig. 5A). We do not understand this unexpected resistance of the Δ$waaY_{inf}$ mutant, but the selective sensitivity of the mutants could have arisen from a differential susceptibility toward the drugs, not from the loss of some defense mechanism toward superoxide in general. To test this hypothesis, we examined whether the *waaY* mutant exhibited differential sensitivity toward superoxide-generating drugs in inducing *soxRS* target genes. For this purpose, the Δ*waaYZ* mutant allele was transduced into a *soxSp-lacZ* reporter strain, MS1343 (17), and the inducibility of LacZ activity was measured. Compared with the wild type, the Δ*waaYZ* mutant became more sensitized to activate the SoxRS system in response to menadione and plumbagin, inducing *soxSp-lacZ* to higher levels at lower concentrations of oxidants (Fig. 5B). This induction was totally dependent on SoxR, since all of the response disappeared by introducing a *soxR* null mutation. However, upon paraquat treatment, there was no difference between the *waaYZ* mutant and the wild type (Fig. 5B). This coincides with no increase in susceptibility to paraquat. These results strongly support the hypothesis that the *waaYZ* genes determine susceptibility toward different drugs, not toward superoxide radical itself.

Changes in outer membrane structure, including LPS. Since WaaY and WaaZ are components of the LPS core synthetic

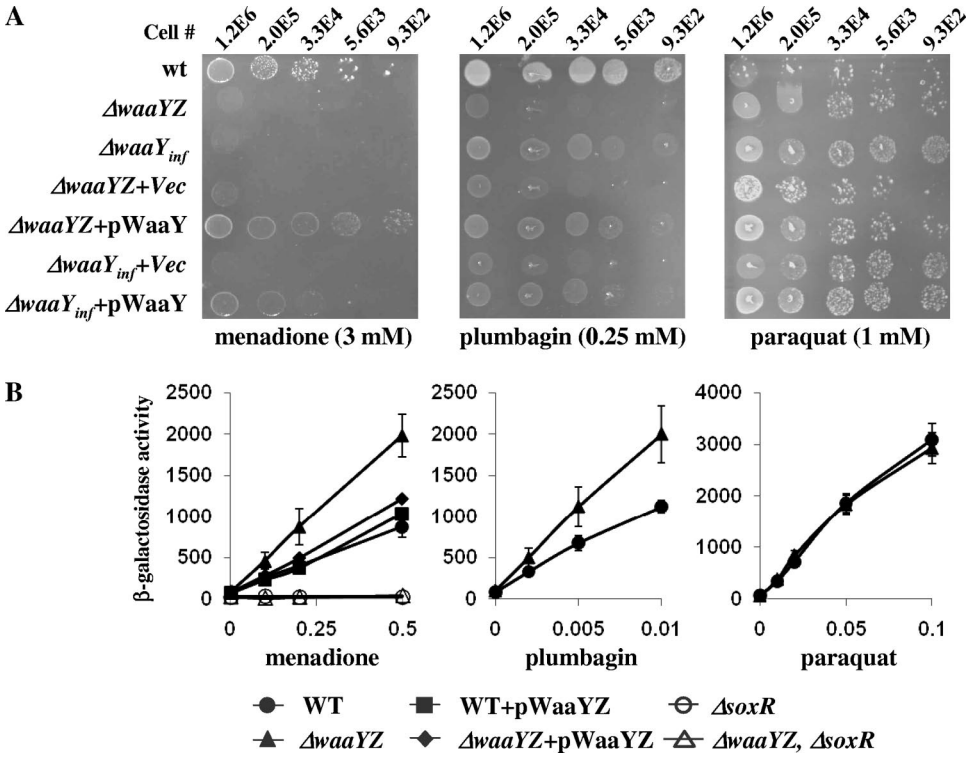

FIG. 5. Sensitivity of *waaY* mutant to superoxide stress. (A) Different amounts of wild-type (GC4468), Δ*waaYZ* (JH1003), and Δ$waaY_{inf}$ cells were grown on LB plates containing superoxide generators for 16 h, and growth was compared. For complementation, a plasmid expressing WaaY (pWaaY) was transformed into the Δ*waaYZ* and Δ$waaY_{inf}$ mutants, and the growth of the transformants was compared. Vec, empty plasmid control. (B) *soxSp-lacZ* fusions in the wild-type (MS1343) and Δ*waaYZ* (JH2003) backgrounds were assayed for β-galactosidase activity to monitor SoxR activity after menadione, plumbagin, and paraquat treatment for 1 h. For complementation, a plasmid carrying *waaYZ* (pWaaYZ) was transformed into the wild-type and Δ*waaYZ* strains, and cells were assayed for β-galactosidase activity. To see the SoxR dependence of the activation, a *soxR* null mutation (BW900) was introduced into both the wild-type and Δ*waaYZ* strains and assayed for β-galactosidase activity.

system, disruption of their genes is likely to cause some alteration in the outer membrane structure, which can be monitored through activation of envelope stress-responsive genes. To do this, the Δ*waaYZ* allele was introduced into a σ^E-dependent reporter strain containing an *rpoH* P3-*lacZ* fusion (11). The *rpoH* P3 promoter is recognized by σ^E, an ECF sigma factor that is activated in response to various forms of extracytoplasmic stresses, including abnormality in LPS (1, 24, 34). Compared with the isogenic parental strain (CAG16037), an about fivefold increase in *rpoH* P3 expression was observed in the Δ*waaYZ* mutant (Fig. 6A), suggesting the presence of envelope stress in the mutant. To determine whether the mutant was indeed altered in LPS structure, we extracted LPSs from the Δ*waaY* and Δ*waaYZ* mutants and analyzed them by SDS-PAGE. LPSs from the mutants showed faster migration, revealing an alteration in LPSs from the mutants (Fig. 6B). These results indicated that WaaYZ-induced modification of LPS structure could serve as a barrier against certain oxidants and drugs. Since WaaY was suggested to be a kinase that phosphorylates heptose II in the LPS inner core (13, 40), a change in phosphorylation status might be one reason for this altered electrophoretic mobility.

WaaYZ confers a protective function against a large number of toxic drugs. If LPS modification serves as a barrier to certain drugs, the *waaYZ* mutant should be sensitive to a wider range of drugs in addition to superoxide generators. Consistently, the *waaYZ* mutant was sensitive to lawsone and some antibiotics, including chloramphenicol and 8-hydroxyquinoline (data not shown). For further examination of this possibility, we performed PM analysis of the *waaYZ* mutant to find out its phenotype under broader growth conditions (9, 42). Among about 2,000 test conditions, including a wide variety of C, N, P, and S sources, a wide pH range, and chemical agents that disrupt various biological pathways, we found that the Δ*waaYZ* mutant showed dramatic sensitivity to a large number of chemicals compared with the reference strain (GC4468). Table 2 summarizes the list of chemicals that exhibited a pronounced growth inhibitory effect on the mutant relative to the wild type (with differential growth values of < -150). It was evident that the Δ*waaYZ* mutant was sensitive to a number of antibiotics and toxic compounds, in addition to redox cycling chemicals. Therefore, WaaYZ-mediated LPS modification confers protection on *E. coli* cells against a wide variety of chemicals not restricted to superoxide generators. We observed that the *waaYZ* mutant became resistant to four antibiotics (bleomycin, phleomycin, dihydrostreptomycin, and neomycin) (not listed in Table 2). This most likely resulted from the presence of the kanamycin/bleomycin resistance cassette in the disrupted *waaYZ* gene (30) and can be regarded as a good positive control for the validity of the PM assay.

The *waaY* promoter is also induced by antibiotics through the MarA system. Since we found that WaaY confers resis-

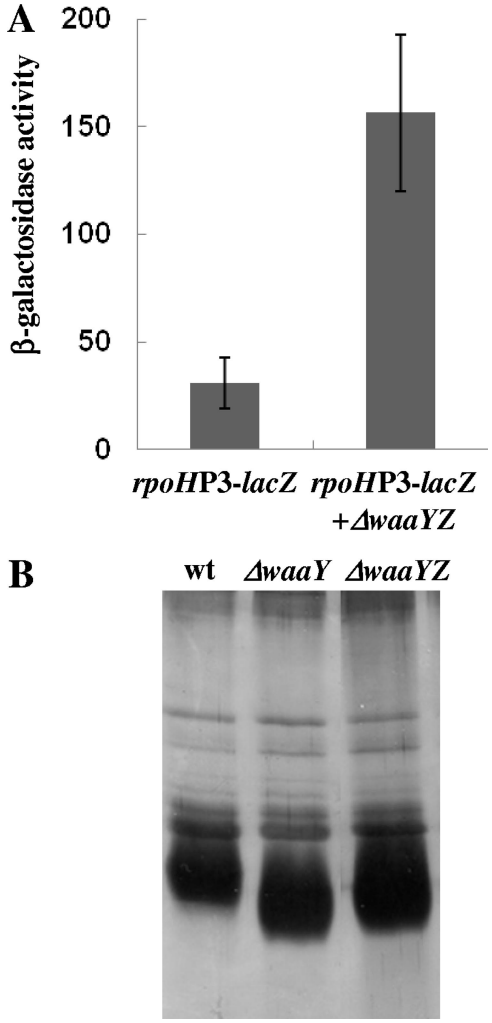

FIG. 6. Changes in outer membrane structure of *waaYZ* mutant. (A) β-Galactosidase activity of the envelope stress reporter strain (*rpoH* P3-*lacZ* single-copy fusion) was assayed in the wild-type background (CAG16037) or Δ*waaYZ* mutant background (JH3003). (B) LPSs were extracted from the wild-type (wt) (GC4468), Δ*waaY* (JH1001), and Δ*waaYZ* (JH1003) strains, separated by 15% SDS-PAGE, and visualized by silver staining.

tance against a wide range of antibiotics, we were intrigued to find out whether the gene is inducible through the MarA system, which responds to various drugs and confers resistance against multiple antibiotics. We examined the effect of sodium salicylate, a known inducer of the *marA* regulon, in the *waaYp-lacZ* reporter strain with a different mutant background. The results in Fig. 7 demonstrate that sodium salicylate also induces the *waaY* gene as efficiently as paraquat does, and the induction is dependent on both the SoxRS and MarA systems. This contrasts with induction by paraquat, which was solely dependent on the SoxS system. We think that this partial SoxRS dependence of salicylate induction could be due to intracellular ROS generation by salicylate treatment, as reported recently (16). When the predicted soxbox sequence was deleted, induction by both paraquat and sodium salicylate was completely gone, indicating that MarA and SoxS activation was mediated through the same *cis*-acting site. Therefore, the *waaY* gene that is induced by superoxide generators (redox cyclers) is also induced by nonredox cyclers through the MarA system and confers resistance toward a wide range of chemicals.

DISCUSSION

It is generally accepted that LPS and the outer membranes of gram-negative bacteria function as a barrier against various antibacterial compounds. However, the mechanism by which this protective function is exerted is relatively less understood. While LPS has an extremely complicated and diverse structure, even in the same species (e.g., *E. coli*), genetic evidence suggests that the barrier property is attributed mainly to lipid A and the core oligosaccharides, since mutations in O-antigen synthesis do not markedly affect membrane integrity or permeability (13, 29). The negatively charged phosphoryl substituents of LPS core oligosaccharides have been postulated as a critical structure to ensure the integrity of the outer membrane through cross-linking of neighboring LPS molecules via binding of divalent cations, thereby conferring resistance to hydrophobic antibiotics and detergents (40, 41). So far, the critical phosphoryl decoration of LPS core in bacteria such as *E. coli*, *Salmonella enterica*, and *Pseudomonas aeruginosa* has been ascribed to the function of *waaP* (formally *rfaP*), whose product phosphorylates HepI (37, 39, 40), and *waaP* mutation has been reported to cause hypersensitivity to novobiocin and SDS (40).

Although the function of *waaY* was also assigned to the phosphorylation of HepII in the LPS inner core, the contribution of *waaY* to the barrier property for resistance has not been addressed. Because the *waaP* activity was prerequisite to the *waaY* function and only mutation of *waaP* resulted in hypersensitivity to novobiocin and SDS (40), little attention has been paid to a role for WaaY. Here we demonstrated that the *waaYZ* locus is particularly induced to function under specific conditions of oxidative stress or antibiotic challenge and apparently plays a role in conferring resistance to a wide range of chemicals and antibiotics under these conditions. *waaY* seems to play a major role in this resistance, possibly through the additional phosphorylation of LPS. However, we do not absolutely rule out the contribution of *waaZ* function, because *waaZ* mutation also causes some susceptibility to drugs. An independent study also showed that the overexpression of WaaZ could make structural changes in LPS, implying that *waaZ* may contribute to LPS structure (12).

While no relationship between oxidative stress and the structural modification of LPS has been reported, *waaY* was recently suggested as a member of the *soxRS* regulon in a genome-wide transcription analysis (8). In addition to the SoxRS-dependent regulation of *waaY* expression, our study demonstrated that the regulation occurred on an internal promoter upstream of *waaY* within the big *waa* operon cluster and that the loss of *waaY* caused a change in LPS structure, which must have been sensed as an extracytoplasmic stress that activated the SigE regulon (Fig. 6A). Therefore, WaaY is an active component in *E. coli* to guarantee the structural integrity of the outer membrane barrier. It echoes the effect of mutation in *gmhD* (formally known as *rfaD*, *htrM*, or *hldD*), encoding an epimerase in LPS biogenesis (Fig. 1A), which also results in envelope stress (23). This coincides with the proposal that

TABLE 2. Compounds toward which the Δ*waaYZ* mutant showed increased sensitivity

Coumpound (fold difference in growth)[a]	Mode of inhibitory action
Small ions	
Potassium tellurite (−274)	Toxic anion
Thallium(I) acetate (−243)	Toxic cation
Cesium chloride (−217)	Toxic cation
Potassium tellurite (−168)	Transport, toxic anion
Sodium nitrite (−157)	Transport, toxic anion
Sodium dichromate (−248)	Transport, toxic anion, SO_4 analog
Chromium chloride (−218)	Transport, toxic cation
Ferric chloride (−163)	Transport, toxic cation
Sodium pyrophosphate decahydrate (−167)	Chelating agent
Antibiotics	
Ethionamide (−267)	Anti-tuberculosis agent
Tinidazole (−172)	Mutagen, nitroimidazole (GP, GN)
Norfloxacin (−247)	DNA topoisomerase, quinolone
Chloroxylenol (−270)	Fungicide
Nordihydroguaiaretic acid (−171)	Lipoxygenase, fungicide
Spectinomycin (−215)	Affects protein synthesis
Oleandomycin (−216)	Affects protein synthesis; 50S ribosomal subunit, macrolide
Fusidic acid (−217)	Affects protein synthesis; elongation factor
Josamycin (−206)	Affects protein synthesis; macrolide
Spiramycin (−157)	Affects protein synthesis; macrolide
Nafcillin (−170)	Affects wall; lactam
Cloxacillin (−169)	Affects wall; lactam
Antiseptics	
9-Aminoacridine (−158)	DNA intercalator
Acriflavine (−151)	DNA intercalator
Agents that affect redox or respiration	
Diamide (−265)	Affects oxidation; glutathione
1-Chloro-2,4-dinitrobenzene (−166)	Affects oxidation; glutathione
Tetrazolium violet (−211)	Affects respiration
Crystal violet (−180)	Affects respiration
18-Crown-6-ether (−152)	Affects respiration; ionophore
Agents that affect membrane functions	
Amitriptyline (−169)	Affects membrane, transport
Guanidine hydrochloride (−224)	Affects membrane; chaotropic agent
Dodecyltrimethyl ammonium bromide (−200)	Affects membrane; detergent, cationic agent
Methyltrioctyl ammonium chloride (−155)	Affects membrane; detergent, cationic agent
Domiphen bromide (−205)	Affects membrane; detergent, cationic, fungicide
Dodine (−187)	Fungicide, guanidine; affects membrane permeability
Other drugs	
Procaine (−246)	Ion channel inhibitor, Na^+ (m)
Promethazine (−179)	Cyclic nucleotide phosphodiesterase
Chlorpromazine (−173)	Phenothiazine
Harmane (−177)	Imidazoline binding sites, agonist
Atropine (−169)	Acetylcholine receptor, antagonist
D,L-Propranolol (−216)	β-Adrenergic blocker
Glycine hydroxamate (−153)	tRNA synthetase
Caffeine (−225)	cyclic AMP phosphodiesterase
5-Azacytidine (−200)	DNA methyltransferase
D-Serine (−176)	Inhibits 3PGA DHase[b] (L-serine and pantothenate synthesis)
D-Cycloserine (−287)	Affects wall, sphingolipid synthesis
Nutrient sources	
Cys-Gly (−193)	N source
Ala-Leu (−178)	N source
Leu-Gly-Gly (−173)	N source
Gly-Gly-Leu (−170)	N source
Leu-Glu (−154)	N source
L-Glutamic acid (−153)	N source
Ala-Leu (−152)	N source
Asp-Leu (−151)	N source
L-Djenkolic acid (−186)	S source

[a] Only high-magnitude differences of ≤−150 are listed here.
[b] 3PGA DHase, 3-phosphoglycerate dehydrogenase.

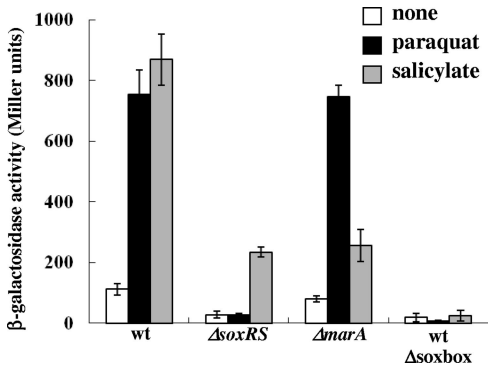

FIG. 7. *waaY* promoter is also induced by antibiotics through the *mar* system. Wild-type single-copy *waaYp-lacZ* fusion cells (JH103) and derivatives of the Δ*soxRS* (JH203) and Δ*marA* (JH603) mutants were treated with paraquat (0.1 mM) and sodium salicylate (10 mM) at early exponential phase (OD_{600} = 0.2), and after 1 h of incubation with vigorous aeration, β-galactosidase activity was assayed. For soxbox deletion in the wild-type background, JH104 was used. We confirmed that the soxbox was correctly deleted and the −35 box remained intact by sequencing.

structural changes in LPS can be sensed by the SigE system, which activates an additional set of genes to cope with external challenges (24). The additional phosphorylation by WaaY should give a significant advantage to the way that WaaP functions, and our PM analysis showed that the *waaYZ* mutant became sensitive to a large number of chemicals.

When we looked into the syntenic organization of the *waa* gene cluster, we found that *waaY* is conserved among most *E. coli* strains, *Salmonella* spp., and *Shigella* spp. but not in *Pseudomonas* spp., where *waaP* is conserved (http://string.embl.de/). Unlike *waaY*, *waaZ* is not conserved in some *E. coli* strains and *Shigella* spp., and its function is not known (13).

The expression of *waaY* is upregulated by both redox-cycling and non-redox-cycling drugs through the SoxRS and MarRA systems. In the previous regulatory model, the expression of *waaP* and *waaY* was regulated by RfaH (SfrB), an antiterminator protein, and this regulation was achieved over the whole transcript from the top promoter in front of *waaQ* (29). In this mechanism, however, even specific induction of the remote genes would demand the extravagant expression of the whole operon. However, our results demonstrate that *waaY* has its own inducible promoter that responds to a wide variety of chemicals and can be expressed specifically as the occasion arises.

It has been revealed that the 'soxbox' (ANNGCAYNNWN NNNCWA) accommodates binding by each of three transcriptional regulators, namely, SoxS, MarA, and Rob (21). The overexpression of Rob also confers resistance against multiple antibiotics and oxidative stress, although the triggering signal of Rob is not yet known (5). Through these multiple regulators, promoters with the soxbox sequence can respond to various stimuli, including multiple antibiotics, generators of superoxide or nitric oxide, and organic solvents (2, 27, 33). The SoxS/MarA/Rob regulon involves ROS scavengers, drug efflux systems, and repair functions (7, 26). Since recent results showed that treatment with many bactericidals produces intracellular ROS by transient depletion of NADH (16), antibiotic-derived induction of ROS scavengers in parallel would provide a great advantage in survival. So far, the induction of drug efflux systems has been regarded as the primary mechanism to confer multiple-drug resistance. However, if the induction of WaaY or WaaYZ by any compound triggering the SoxS/MarA/Rob system causes a modification of LPS capable of inducing resistance, it might serve as a reinforced barrier against other drugs. Therefore, strengthening of the outer membrane barrier against diverse drugs is another clever way of ensuring multiple-drug resistance. This strategy would be extremely beneficial for pathogenic bacteria to escape the oxidative attack by the immune system, which is often accompanied by treatment with chemotherapeutic drugs. Since WaaY can be induced by any oxidative stress and by drugs, we suggest that bacteria use WaaY as a common tool to deal with two stresses and to confer resistance during the infectious process, as it can efficiently meet attacks by both stresses.

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