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The *Escherichia coli* Azoreductase AzoR Is Involved in Resistance to Thiol-Specific Stress Caused by Electrophilic Quinones[▽]

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The physiological role of *Escherichia coli* azoreductase AzoR was studied. It was found that AzoR was capable of reducing several benzo-, naphtho-, and anthraquinone compounds, which were better substrates for AzoR than the model azo substrate methyl red. The Δ*azoR* mutant displayed reduced viability when exposed to electrophilic quinones, which are capable of depleting cellular reduced glutathione (GSH). Externally added GSH can partially restore the impaired growth of the Δ*azoR* mutant caused by 2-methylhydroquinone. The transcription of *azoR* was induced by electrophiles, including 2-methylhydroquinone, catechol, menadione, and diamide. A transcription start point was identified 44 bp upstream from the translation start point. These data indicated that AzoR is a quinone reductase providing resistance to thiol-specific stress caused by electrophilic quinones.

Azo dyes are organic colorants characterized by the presence of one or more azo groups. They are used widely in textile, printing, cosmetics, pharmaceutical, food, and other industries because of their ease of synthesis and chemical stability (35). In addition, azo compounds are also the most commonly used drugs in the treatment of inflammatory bowel disease (20, 46). However, the release of these compounds into the environment is undesirable, not only because of their color, but also because many azo compounds and their breakdown products are toxic and/or mutagenic (7, 48, 49). Biological treatment of azo dyes by the use of bacteria has been studied widely recently (14, 41).

Enzymes that catalyze the reduction of azo groups are termed azoreductases. Utilizing NADH and/or NADPH as an electron donor, azoreductase can decolorize azo dyes into corresponding aromatic amines by reductive cleavage of azo bonds. The decolorization was regarded as the rate-limiting step, which was followed by the aerobic mineralization of the colorless aromatic amines (6, 7, 41). Furthermore, azoreductase is also involved in the site-specific delivery of azo prodrugs, which are therapeutically inactive in their intact form and rely on azo reduction by azoreductases of intestinal microflora for activation (20, 46). Proteins with azoreductase activity have been identified and characterized from a wide variety of bacteria, such as *Pigmentiphaga kullae* K24, *Xenophilus azovorans* KF46F, *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus* sp. strain OY1-2, *Pseudomonas aeruginosa*, and *Rhodobacter sphaeroides* (2, 3, 8, 9, 31, 42, 46, 50). There are at least two different types of bacterial azoreductases: those that require flavin and those that do not (7). Flavin-dependent azoreductases can be further classified into two families according to their amino acid sequences (9, 28). Azoreductases from *E. coli* and *Bacillus* sp. strain OY1-2 are representative of the two flavin-dependent azoreductase families, respectively. Although they are effective for an in vitro enzyme assay, overexpression of these azoreductases in vivo results in little or no increase of bacterial decolorization activity (3, 39). Thus, the physiological role of azoreductase has recently been a subject of debate. As the introduction of azo compounds into the environment is due mainly to human activities, reduction of azo dyes may not be the primary roles of these enzymes.

Initially, acyl carrier protein phosphodiesterase of *E. coli*, encoded by *acpD*, was partially purified, and its N-terminal sequence was obtained (17). Further study demonstrated that AcpD is a flavin mononucleotide (FMN)-dependent NADH-azoreductase having no acyl carrier protein phosphodiesterase activity. It catalyzes the reductive cleavage of the azo bond in methyl red via a ping-pong mechanism. The *acpD* gene was thus redesignated *azoR* (31). The crystal structure of oxidized AzoR was determined to be a homodimeric FMN-containing enzyme. Each of its monomers has a flavodoxin-like structure (22). Further studies of three-dimensional structures and site-directed mutagenesis resulted in an authentic AzoR-methyl red complex model and suggestions of probable molecular mechanism (23). However, the physiological function of AzoR still remains unknown. AzoR homologs are widely distributed in both gram-positive and gram-negative bacteria, represented by *E. faecalis* AzoA and *E. coli* AzoR, respectively (7, 8, 23). The lateral transfer of the azoreductase gene among enteric bacteria was suggested based on phylogenetic analysis (1). Recently, the *yvaB* gene of *Bacillus subtilis* was reported to be regulated by repressor YkvE in response to thiol-specific stress (43). YvaB has 28% similarity in amino acid sequence with AzoR, and its azoreductase activity was poorly profiled (33). The *yocJ* gene of *B. subtilis*, a paralogue of *yvaB*, encodes

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
E. coli strains		
JM109	*endA1 recA1 gyrA96 thi-1 hsdR17 relA1 supE44* Δ(*lac-proAB*)	Promega
ADH	JM109 pKD46	This study
LG1	JM109 *azoR*::*kan* Km^r	This study
LG2	JM109 pGEX-azoR	This study
Plasmids		
pGEX-4T-1	Overexpression vector with GST tag; Amp^r	Promega
pGEX-azoR	pGEX-4T-1 containing *azoR* fragment from *E. coli* JM109; Amp^r	This study
pKD46	Red recombinase-expressing plasmid; Amp^r	12
pKD4	FRT-flanked kanamycin cassette template	12

[a] Km^r, kanamycin resistance; Amp^r, ampicillin resistance.

protein YocJ, which possesses 29% amino acid sequence similarity with AzoR. Although characterization of its enzyme activity is not available, YocJ was proposed to be involved in quinone detoxification (24).

Quinones are found widely in nature and are metabolites or products of different organisms (13). They can participate in deleterious redox cycling, which leads to the accumulation of reactive oxygen species. Intracellular production of reactive oxygen species, such as superoxide, H_2O_2, and hydroxyl radical, can impair lipids, proteins, and nucleotides (13, 34). On the other hand, electrophilic quinones can form *S*-adducts through covalent bonds with cellular thiols by Michael addition, which depletes cellular thiols and causes thiol-specific stress in cells (37).

In this study we show that the quinone reductase activity of AzoR is essential for the normal growth of *E. coli* exposed to electrophilic quinones. Electrophilic quinones can result in the elimination of cellular reduced glutathione (GSH). The transcription of *azoR* is induced by 2-methylhydroquinone (2-MHQ), catechol, menadione, and diamide. The regulation of *azoR* transcription may be different from those of *yvaB* and *yocJ* of *B. subtilis*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *E. coli* strains and plasmids used in this study are listed in Table 1. They were grown aerobically at 37°C in Luria-Bertani (LB) medium.

Construction of *azoR* knockout mutant. The one-step replacement method described by Datsenko and Wanner (12) was used to construct an *azoR* deletion in *E. coli* JM109. PCR was done using pKD4 as a template and primers D1 (5′-TGGACTGAATATCTACAGTCCACATCAAGACCGTGTCCGGCATATGAATATCCTCCTTAG-3′) and D2 (5′-AACGGGGCATCGCCCATTCAAACATCTATAAGGAAACACCGTGTAGGCTGGAGCTGCTTC-3′). The underlined sequences anneal to the template plasmid, while the remaining sequences correspond to the ends of *azoR*. The PCR-amplified DNA fragment was electroporated into *E. coli* ADH containing plasmid pKD46, which expresses Red recombinase and was cured later by growth at 37°C. The mutant strain *E. coli* LG1 was selected on an LB plate supplemented with kanamycin. The deletion of the gene was confirmed by PCR and sequencing.

Cloning of *azoR* from *E. coli* JM109. The *azoR* gene was PCR-amplified from the chromosomal DNA of *E. coli* JM109 by using primers P1 (5′-GAATTCATGAGCAAGGTATTAGTTCTTAA-3′) and P2 (5′-CTCGAGTTATGCAGAAACAATGCTGTCG-3′). The amplified DNA fragment was digested with EcoRI and XhoI and cloned into the EcoRI/XhoI double-digested pGEX-4T-1. The resulting plasmid pGEX-azoR was transformed into *E. coli* JM109, and the resultant *E. coli* LG2 was used for the expression of GSH *S*-transferase (GST) fusion AzoR protein.

Purification and reductase activity assay of AzoR. *E. coli* LG2 cells were grown in LB medium to an optical density at 600 nm (OD_{600}) of 0.5, and then the *azoR* gene was induced by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside to the cultures and further incubating them for 3 additional hours. Cells were then harvested by centrifugation at 4,000 × *g* for 15 min and washed twice with 20 mM sodium phosphate buffer, pH 7.0. The pellets were frozen at −80°C overnight. The cells that were thawed on ice and resuspended in phosphate buffer were disrupted by ultrasonication three times for 20 s each time and spun at 50,000 × *g* for 20 min at 4°C. The supernatant fraction was saved. The GST-AzoR protein was purified by glutathione Sepharose 4B (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The GST tag was removed from AzoR by digestion with 10 U of thrombin per mg of fusion protein. It was mixed gently and incubated in cleavage buffer (20 mM Tris-HCl [pH 8.5], 120 mM NaCl, and 2.5 mM $CaCl_2$) at room temperature (22 to 25°C) for 16 h. The thrombin-treated samples were purified again with glutathione Sepharose 4B according to the manufacturer's instructions. The progress of AzoR purification was monitored by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The protein concentration was determined by the method of Bradford (5), with bovine serum albumin as a standard.

The standard reductase activity assay system comprised 20 mM sodium phosphate buffer (pH 7.0), 10 to 700 μM electron acceptor substrate (methyl red or various quinone compounds), 100 μM NADH, 20 μM FMN, and a suitable amount of enzyme. The reaction was initiated by the addition of NADH. Reductase activities were monitored based on the oxidation of NADH at 340 nm ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$) (26). Values of K_m (μM) and k_{cat} (s^{-1}) were obtained from Lineweaver-Burk plots, and k_{cat}/K_m ($s^{-1}\ mM^{-1}$) values were calculated.

Effects of stressors on growth of *E. coli* strains. The overnight cultures of *E. coli* JM109 and *E. coli* LG1 were used to inoculate fresh LB medium to a starting OD_{600} of 0.1. They were grown at 37°C aerobically until the OD_{600} reached 0.6. Then each culture was split equally into several portions and treated with stressors, including 0.5 mM 2-MHQ, 1 mM diamide, 6 mM catechol, 0.3 mM menadione, and 1 and 10 mM H_2O_2. Intracellular GSH measurements were made using a GSH assay kit (Calbiochem, San Diego, CA).

To study the effects of exogenous GSH on the growth of *E. coli* JM109 and *E. coli* LG1 in the presence of 2-MHQ, 0.25 to 2 mM GSH was added to the cultures before the addition of 0.5 mM 2-MHQ.

Quantitative PCR analysis. *E. coli* JM109 culture was grown at 37°C aerobically, and samples were taken in different growth phases to study the transcription of *azoR* under normal growth conditions.

Effects of different stressors on the transcription of *azoR* were evaluated. Various stressors were applied to *E. coli* cultures when the OD_{600} reached 0.6. Samples were taken before (0 min) and 10 and 20 min after addition of stressors.

Total RNA was obtained with RNAiso reagent (TaKaRa Dalian). DNA contamination was eliminated by a DNase I treatment at 37°C for 1 h. RNA integrity was checked by agarose gel electrophoresis. The concentration and purity of extracted RNA were determined by measuring the A_{260} and A_{280}. In total, 0.5 μg of purified RNA was reverse transcribed (15 min at 37°C and 5 s at 85°C) by using PrimeScript real-time (RT) enzyme mix I (TaKaRa Dalian). For the reverse transcription, 50 μM Oligo dT primer and 100 μM random hexamers were used in each reaction. Quantitative RT-PCR was performed with a TaqMan assay in a Thermal Cycler Dice RT system (TaKaRa Dalian) using the following conditions: 10 s at 95°C and 45 cycles of 5 s at 95°C and 30 s at 60°C. The primers used for amplification of *azoR* cDNA were azoR-F (5′-GGGATCCACAAAGATGGACCAAC-3′) and azoR-R (5′-GGCGAAGACAAATTTCACATCGG-3′), and the TaqMan probe was azoR-P (5′-[FAM]-ACCTGGTGACGCCGTATCTGTCCACG-[Eclipse]-3′). *E. coli* 16S rRNA was chosen as an endogenous control for normalization of the cDNA loading in each PCR. Primers used for amplification of 16S rRNA cDNA were 16S rRNA-F (5′-CCATGAAGTTGGAATCGCTAG-3′) and 16S rRNA-R (5′-GCTTGACGGGCGGTGT-3′), and the TaqMan probe was 16S rRNA-P (5′-[FAM]-TACAAGGCCCGGGAACGTATTCACCG-[Eclipse]-3′). The reaction mixtures (25 μl) contained 12.5 μl of Premix Ex *Taq* (2×) (TaKaRa Dalian), 10 μM each primer, 3 μM TaqMan probe, and 2-μl portions of different dilutions of the reverse transcription product. The standard curves for quantification were calculated by serial 10-fold dilutions of the standard cDNA transcript. Control reaction in which reverse transcriptase was omitted from the reaction mixture ensured that DNA products resulted from the amplification of cDNA rather than from DNA contamination.

Determination of TSP. RNA was extracted from cells exposed to 2-MHQ for 10 min. Determination of the transcription start point (TSP) was performed

TABLE 2. Quinone reductase activities of AzoR[a]

Substrates	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
2-Methyl-1,4-benzoquinone	1,704.5 ± 26.3	72.0 ± 3.5	42.2
2-Hydroxy-1,4-naphthoquinone	87.3 ± 2.4	5.8 ± 0.4	66.4
Anthaquinone-2-sulfonate	8.1 ± 0.6	1.1 ± 0.3	135.8
Antraquinone-2,6-disulfonate	14.6 ± 0.8	2.6 ± 0.2	178.1

[a] Values of kinetic parameters were obtained from Lineweaver-Burk plots. Data (means ± standard deviations) were from three independent experiments.

using the 5′-Full rapid amplification of cDNA ends (RACE) kit (TaKaRa Dalian) according to manufacturer's instructions. The adaptor-ligated cDNA was amplified by two sequential reactions using *azoR* outer primer (5′-CATCG CTCGGACGCAGA-3′) and *azoR* inner primer (5′-CAACCAGTTCGCCATC CAGTA-3′). After denaturation at 94°C for 3 min, the amplification (30 cycles) was 98°C for 15 s, 52°C for 30 s, and 72°C for 2 min, followed by one cycle of 72°C for 10 min. The PCR products were sequenced to recognize the relevant TSP.

Sequence alignment and structure analysis. Multiple sequence alignment was conducted using Clustal X 1.8 (10) and visualized with Genedoc 2.6 (32). Three-dimensional structure analysis was conducted using PyMol 0.99 (DeLano Scientific, San Carlos, CA).

Statistical analysis. Student's *t* test was employed to investigate statistical differences. Samples with *P* values of <0.05 were considered statistically different.

RESULTS

Sequence and structure analysis. The amino acid sequence of AzoR was aligned with those of other proteins possessing azo and/or quinone reductase activity, including ChrR of *Pseudomonas putida*, WrbA of *E. coli*, NQO1 of *Homo sapiens*, Lot6p of *Saccharomyces cerevisiae*, YvaB of *B. subtilis*, and AZR of *R. sphaeroides* (19, 34, 38, 40, 43, 50). The sequence length varied, and the overall sequence similarity was not obvious. However, high similarities were found in the middle parts of their sequences (Pro95-Gly148 of AzoR). Residues Tyr96, Asn97, Phe98, Gly141, and Gly142 of AzoR, which are involved in the binding of FMN (22), were relatively conserved in these quinone reductases. Structure studies have shown that AzoR, NQO1, and AZR all possess similar flavodoxin-like α/β cores (22, 25, 26). All these conserved residues are located in similar loops around the flavin cofactors.

Reductase activity. Flavoenzymes usually act on a wide range of substrates that vary greatly in chemical nature and size (18). Using NADH as an electron donor, AzoR was reported to exploit some azo dyes and menadione as electron acceptors (31). However, no further information about its substrate spectrum was available. As shown in Table 2, AzoR could use 2-methyl-1,4-benzoquinone, 2-hydroxy-1,4-naphthoquinone, anthraquinone-2-sulfonate, and anthraquinone-2,6-disulfonate as electron acceptors. According to the k_{cat}/K_m value, anthraquinone-2,6-disulfonate was the best substrate for AzoR among the quinones examined. These quinones have higher k_{cat}/K_m values than methyl red (15.5 s^{-1} mM^{-1}), which has been used as a model azo substrate in previous studies of azoreductase activity and the three-dimensional structure of AzoR (23, 31). Poor activity was detected with the thiol oxidant diamide containing an azo bond (data not shown). AZR of *R. sphaeroides* can also function as nitroreductase (26). However, no nitroreductase activity was detected with AzoR toward nitrofurazone and 2,4,6-trinitrotoluene. NADH could not be replaced with NADPH, and no activity was detectable without externally added FMN (data not shown).

The Δ*azoR* mutant is sensitive to thiol-specific stress caused by quinones. Cultures of *E. coli* JM109 and *E. coli* LG1 were treated with various stressors. 2-MHQ and catechol can readily

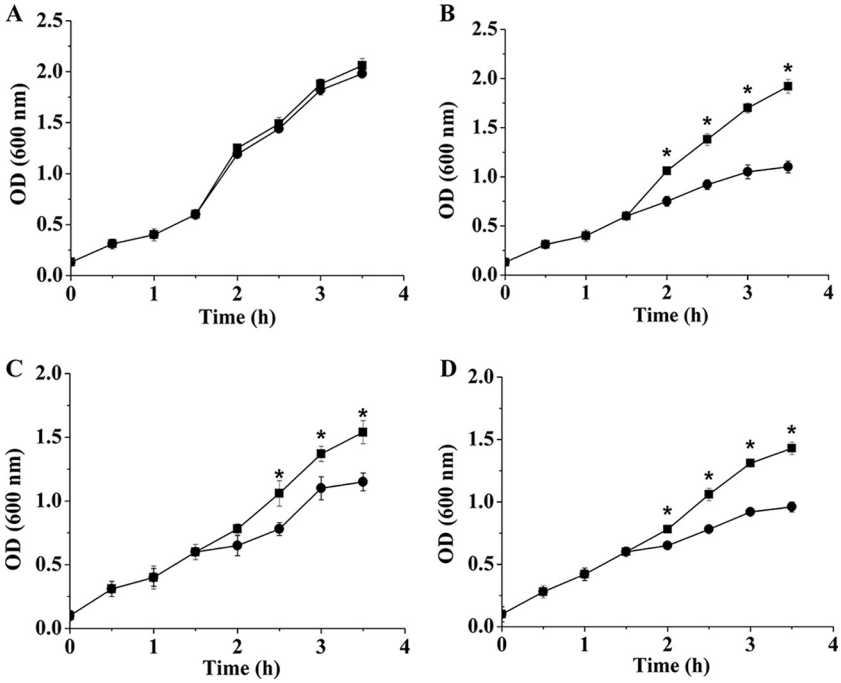

FIG. 1. Aerobic growth of *E. coli* JM109 (■) and the *E. coli* LG1 Δ*azoR* mutant (●) with no treatment (A) and after exposure to 0.5 mM 2-MHQ (B), 6 mM catechol (C), and 0.3 mM menadione (D). Data (means ± standard deviations) were from three independent experiments. Statistically significant differences ($P < 0.05$) were determined by Student's *t* test and are indicated with an asterisk.

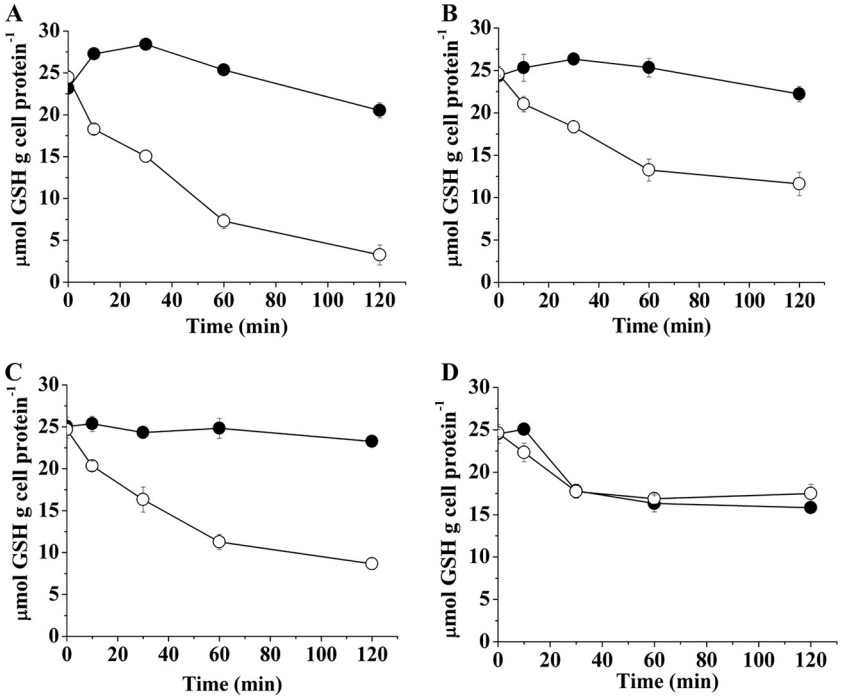

FIG. 2. Effects of addition of 0.5 mM 2-MHQ (A), 6 mM catechol (B), 0.3 mM menadione (C), and 1 mM diamide (D) on cellular GSH concentrations of *E. coli* JM109 (●) and *E. coli* LG1 (○). Data (means ± standard deviations) were from three independent experiments.

be oxidized to corresponding benzoquinones under aerobic conditions (24). As shown in Fig. 1, no obvious growth difference between the two strains was observed when no treatment was applied. The growth of the Δ*azoR* mutant was impaired after treatment with 0.5 mM 2-MHQ, 6 mM catechol, or 0.3 mM menadione. No obvious growth difference between the two strains was found after treatment with 1 mM thiol-oxidizing diamide or H_2O_2 (data not shown).

The effects of electrophiles on cellular GSH levels of the two *E. coli* strains were evaluated (Fig. 2). When no treatment was applied, the cellular GSH levels of the two strains were unchanged in 2 h (data not shown). The cellular GSH levels of the Δ*azoR* mutant decreased around 87%, 65%, and 53% in 2 h after the addition of 2-MHQ, menadione, and catechol, respectively, but those of *E. coli* JM109 were relatively stable. When lower concentrations of the stressors were added, the cellular GSH level showed a smaller decrease, and less growth inhibition was observed (data not shown). *S*-adduct might be formed via addition of GSH (Michael-type addition) to these electrophilic quinones. In addition, the cellular GSH levels of the two strains decreased similarly after exposure to diamide.

The Δ*azoR* mutant was partially relieved from impaired growth caused by 2-MHQ when exogenous GSH was added to the culture (Fig. 3). However, no further protection effect was observed when more than 0.5 mM GSH was added (data not shown). In addition, the growth of *E. coli* JM109 in the presence of 2-MHQ was not affected by externally added GSH (data not shown).

Effect of stressors on *azoR* transcription. Transcription of *azoR* was analyzed using quantitative RT-PCR. Under normal growth conditions, there is no significant dependence of transcription on growth phase (Fig. 4). Then the effect of stressors on *azoR* transcription was investigated using 0.5 mM 2-MHQ, 1 mM diamide, 6 mM catechol, 0.3 mM menadione, and 1 and 10 mM H_2O_2. As shown in Table 3, the transcription of *azoR* was induced significantly in the presence of 2-MHQ, catechol, or menadione. Exposure to diamide also resulted in around 10-fold induction of *azoR* transcription. However, the addition of 1 mM H_2O_2 had no effect on *azoR* transcription. At a higher concentration of H_2O_2 (10 mM), a two- or three-fold increase in the *azoR* transcript was observed.

TSP of *azoR*. Although both YocJ and YvaB of *B. subtilis* show around 30% amino acid sequence identity with AzoR, no homologous gene was found in upstream and downstream sequences of *azoR*, *yocJ*, and *yvaB*. Using total RNA of cells

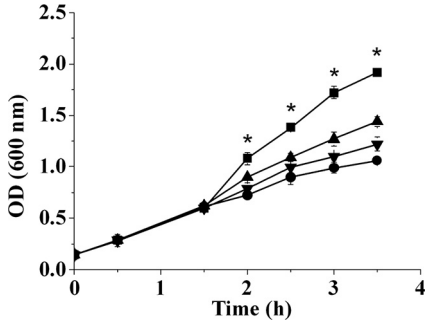

FIG. 3. Aerobic growth of *E. coli* JM109 (■) and the *E. coli* LG1 Δ*azoR* mutant in the presence of externally added GSH (●, 0 mM; ▼, 0.25 mM; ▲, 0.5 mM) after exposure to 0.5 mM 2-MHQ. Data (means ± standard deviations) were from three independent experiments. Statistically significant differences ($P < 0.05$) were determined by Student's *t* test and are indicated with an asterisk.

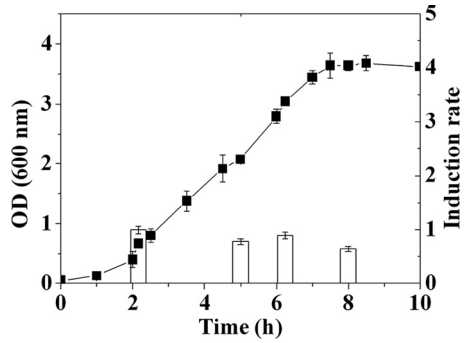

FIG. 4. Growth (■) and *azoR* transcription level (white bars) of *E. coli* JM109 under normal conditions.

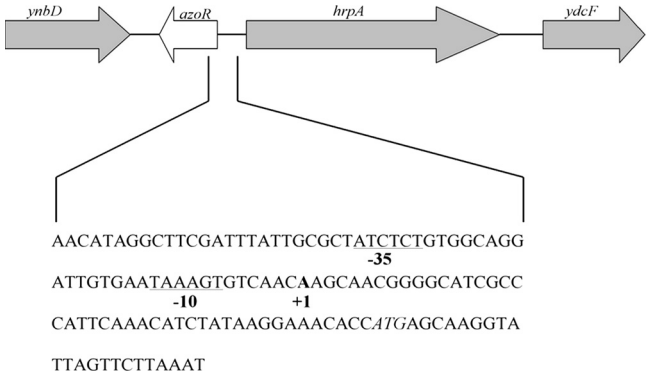

FIG. 5. Schematic representation of the *azoR* locus and analysis of its TSP. Open reading frames are represented by block arrows, and their orientations indicate the transcriptional direction. The upstream sequence of the *azoR* open reading frame is shown below the diagram. The TSP (+1) determined by 5′ RACE after exposure to 2-MHQ is in bold. The putative −10 and −35 boxes are underlined. The start ATG codon is shown in italics.

exposed to 2-MHQ, the TSP of *azoR* was determined with 5′ RACE. Transcription of *E. coli azoR* was initiated from a TSP 44 bp upstream of the translation start point (Fig. 5).

DISCUSSION

Most azo compounds are generally very polar and/or large molecules, which render them difficult to diffuse through the cell membrane. Moreover, the reduction products of azo compounds from azoreductases (i.e., corresponding aromatic amines) are usually more toxic than azo substrates. Thus, intracellular azoreductase should play a physiological role different from reduction of azo compounds.

Around 40 to 50 amino acids involved in the binding of substrates and flavin cofactors (23, 25) were found conserved in the middle parts of the amino acid sequences of AzoR and other quinone reductases. Thus, the quinone reductase activity of AzoR was investigated. It can reduce several benzo-, naphtho-, and anthraquinone compounds. Enhanced values of catalytic efficiency (k_{cat}/K_m) were observed as the number of the fused rings increased from one to three. NfsB of *E. coli* can also reduce quinone compounds with one to three fused rings. However, benzo- and naphthoquinones were found to be much better substrates than anthraquinones (36). ChrR of *P. putida* and WrbA of *E. coli* and *Archaeoglobus fulgidus* were reported to reduce benzo- and naphthoquinone compounds (19, 34). Lot6p of *S. cerevisiae* can utilize 1,4-benzoquinone and 1,4-naphthoquinone, but not 1,4-anthraquinone, as substrate (40). AZR of *R. sphaeroides* can reduce several naphthoquinone and anthraquinone compounds, but it cannot utilize 1,4-benzoquinone as substrate (27). Many quinone reductases can use both NADH and NADPH as sources of electrons, with a preference for either NADH (e.g., ChrR and WrbA [19, 34]) or NADPH (e.g., AZR and Lot6p [27, 40]), while AzoR can use only NADH. Externally added FMN is indispensable for the reductase activity of AzoR but not required for the activities of other quinone reductases.

TABLE 3. Induction of *azoR* transcription by different stressors investigated with quantitative RT-PCR

Stressor	Induction rate[a]	
	10 min	20 min
0.5 mM 2-MHQ	46.0 ± 1.2[b]	129.1 ± 4.5[b]
6 mM catechol	15.6 ± 3.4[b]	31.9 ± 2.6[b]
0.3 mM menadione	25.5 ± 2.2[b]	13.3 ± 3.1[b]
1 mM diamide	11.4 ± 0.9[b]	8.1 ± 2.5[b]
1 mM H_2O_2	0.9 ± 0.2	0.6 ± 0.3
10 mM H_2O_2	3.0 ± 1.1	1.9 ± 0.7
No stressor	1.1 ± 0.2	1.2 ± 0.1

[a] The increase in expression of *azoR* mRNA at 10 min and 20 min is calculated relative to the expression at zero time before stress is applied. Data (means ± standard deviations) were from three independent experiments.

[b] Samples showing significant differences which were determined by Student's *t* test ($P < 0.05$).

Some quinone reductases were reported to be involved in H_2O_2 resistance (19, 21, 27, 47). However, no growth difference was detected between *E. coli* JM109 and the Δ*azoR* mutant after exposure to H_2O_2. Furthermore, the Δ*azoR* mutant showed impaired growth in the presence of electrophilic quinones, such as 2-MHQ, catechol, and menadione. Electrophiles can severely damage biological nucleophiles, such as the bases of DNA and the thiol groups of proteins (30). GSH is the major low-molecular-weight thiol in *E. coli* cells. It can interact with toxic dicarbonyl compounds, such as methylglyoxal and *N*-ethylmaleimide, resulting in the formation of GSH-*S*-adduct as a mechanism for detoxification (GSH-dependent detoxification) (4, 15, 16). It was shown that AzoR is essential for the maintenance of cellular GSH after addition of 2-MHQ, catechol, and menadione. Thus, instead of defending against general oxidative stress, AzoR is involved in resistance to thiol-specific stress. Exogenous GSH was reported to protect some bacteria against various stresses (44, 45, 51). It was found that externally added GSH can partially restore impaired growth of the Δ*azoR* mutant caused by 2-MHQ but cannot achieve complete restoration. Thus, these electrophilic quinones may be involved in the depletion of other essential cellular thiolates besides GSH. The Δ*yocJ* mutant of *B. subtilis* shows no growth defect after exposure to catechol, 2-MHQ, and diamide. However, the Δ*yvaB* mutant of *B. subtilis* is sensitive to catechol and 2-MHQ. Moreover, the Δ*yvaB* Δ*yocJ* double mutant is more sensitive to catechol and 2-MHQ compared with the Δ*yvaB* single mutant, suggesting that YvaB can complement YocJ deficiency in response to detoxification (24, 43). As *B. subtilis*

does not possess GSH, it was suggested that other protein thiolates and nonprotein low-molecular-weight thiolates might be involved in detoxification of electrophiles (24). The Δ*yvaB* Δ*yocJ* mutant shows impaired growth in the presence of 1 mM diamide (24). However, exposure to diamide results in no difference in the growth and cellular GSH levels between *E. coli* JM109 and the Δ*azoR* mutant. Thus, AzoR is not essential for resistance to diamide. There may be other proteins involved in diamide detoxification in *E. coli*.

The transcription of *E. coli azoR* is induced by exposure to electrophilic quinones and diamide. Remarkably, the transcription level is induced almost 130 times higher by 2-MHQ in 20 min. Thus, cells of *E. coli* are protected against thiol-specific stress caused by electrophilic quinones by elevated expression of AzoR. The transcription level of the *chrR* gene encoding quinone reductase ChrR is increased 10 times when challenged with 1 mM H_2O_2 (19). However, the addition of 10 mM or 1 mM H_2O_2 results in little or no increase of the *azoR* transcript. Northern blot experiments demonstrated that the transcriptions of *B. subtilis yocJ* and *yvaB* are also induced by catechol, 2-MHQ, and diamide. And there is no induction of *yocJ* and *yvaB* transcription in response to oxidative stress caused by 1 mM H_2O_2 (24, 43).

The neighboring genes, TSP, and putative promoter region of *azoR* show no similarity with those of *yocJ* and *yvaB*. It was reported that *yocJ* and *yvaB* are negatively regulated by MarR-type repressors YkvE and YodB, respectively (24, 43). However, our analysis of the genome of *E. coli* revealed that it does not contain genes homologous to *ykvE* and *yodB*. Moreover, the binding sites of the *E. coli* MarR repressor (11, 29) were not found in the promoter region of *azoR*. Thus, homologous AzoR proteins of gram-positive and gram-negative bacteria may be regulated in different ways, which warrants further investigation.

Our studies also found no difference in the decolorization abilities between *E. coli* JM109 and the Δ*azoR* mutant (data not shown). Similar results were obtained in the study of an *azoR* homolog from *Shewanella oneidensis* (6). In conclusion, the well-studied azoreductase AzoR has no contribution to dye reduction in vivo and is actually a quinone reductase playing a role in protection against thiol-specific stress.

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