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SoxRS-Regulated Expression and Genetic Analysis of the *yggX* Gene of *Escherichia coli*

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Genomic studies with bacteria have identified redox-responsive genes without known roles in counteracting oxidative damage. Previous transcriptional profiling showed that expression of one such gene, *yggX*, was activated by superoxide stress in *Escherichia coli*. Here we show that this activation could be mimicked by artificial expression of the regulatory protein SoxS. Northern analysis confirmed the transcriptional activation of *yggX* by oxidative stress or SoxS expression but not in response to the related MarA or Rob proteins. Northern analysis showed that *mltC*, which codes for a peptidoglycan hydrolase and is positioned immediately downstream of *yggX*, was also regulated by oxidative stress or ectopic expression of SoxS. Purified SoxS protein bound to the predicted *yggX* promoter region, between positions 223 and 163 upstream from the *yggX* translational start site. Within this region, a 20-bp sequence was found to be necessary for oxidative stress-mediated activation of *yggX* transcription. A *yggX* deletion strain was hypersensitive to the redox-cycling agent paraquat, and a plasmid expressing YggX complemented the sensitivity of the deletion strain. Under exposure to paraquat, the *yggX* deletion strain showed a deficiency in aconitase activity compared to the isogenic wild-type strain, while expression of YggX from a multicopy plasmid increased the aconitase levels above those of the wild-type strain. These results demonstrate the direct regulation of the *yggX* gene by the redox-sensing SoxRS system and provide further evidence for the involvement of *yggX* in protection of iron-sulfur proteins against oxidative damage.

Cellular responses to environmental stress involve concerted changes in the expression of multiple genes. Recently developed genomic techniques such as transcriptional profiling have allowed the identification of hundreds of stress-responsive genes, including many lacking a known function or significant homology to genes with known functions. In particular, the genetic response of *Escherichia coli* to oxidative stress includes dozens of such genes (39, 52). The observation that some of these uncharacterized genes are under the control of known oxido-responsive signal transduction systems suggests that they might have direct roles in the antioxidant response (39, 52).

The *yggX* gene of *E. coli* was identified as an open reading frame with a predicted 11-kDa protein product during genomic sequencing (7), and further protein expression studies demonstrated that the gene codes for a small and abundant protein (29, 49). Sequence comparisons revealed that *yggX* homologs are conserved through gram-negative bacteria and that the structural organization of three neighboring genes is conserved (15). Moreover, the *yggX* gene is transcribed as part of a complex operon that may direct several transcripts containing different combinations of structural genes (Fig. 1).

The first report on YggX function showed that overexpression of the *Salmonella enterica* YggX protein complements the thiamine dependence of a *gshA* (glutathione-deficient) *Salmonella* strain, probably by restoring the function of thiamine-synthetic enzymes affected by increased oxidant levels (16). Overexpression of YggX also enhances *Salmonella*'s resistance to oxidants, reduces oxidant-induced mutagenesis, and restores the aconitase deficiency of a *gshA* mutant. Hence, Gralnick and Downs have proposed that YggX protects FeS clusters in biosynthetic enzymes from oxidative damage (16). Recently, the same authors have shown that YggX decreases chelatable iron in solution and protects DNA from iron-mediated oxidative damage (16a).

Transcriptional profiling experiments (39) showed that *yggX* is activated as part of *E. coli*'s response to superoxide stress under the control of SoxRS, a well-characterized signal transduction system (see reference 41 for a review). In the SoxRS regulatory cascade, SoxR senses oxidative stress in the cytoplasm via the oxidation state of its Fe-S cluster (24, 50). When oxidized, SoxR activates the transcription of *soxS*, and the resulting SoxS protein activates genes that collectively help to avoid or repair the damage caused by oxidants (13, 18, 19). SoxS binds as a monomer to sites resembling the asymmetric, degenerate consensus AYnGCACnnWnnRYYAAAYn (where n is any base, Y is T or C, W is A or T, and R is A or G) (33). SoxS-activated genes with known functions include *sodA* (Mn superoxide dismutase), *acnA* (aconitase A), *fpr* (ferredoxin oxidoreductase), and *fur* (Fe-binding transcriptional repressor).

In this report we show that the transcriptional activation of the *E. coli yggX* gene under oxidative stress is mediated directly by SoxS and provide further evidence for its role in the cellular defense against oxidation.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1.

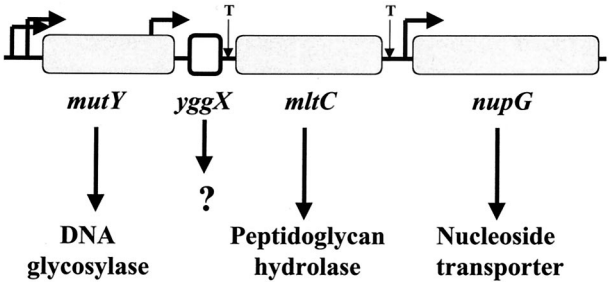

FIG. 1. Structure of the *mutY-yggX-mltC-nupG* region. Schematic representation of the *yggX* region, after that described previously (15). The horizontal arrows represent promoters, and the thin vertical arrows show the approximate positions of proposed termination sites (T) that allow transcript readthrough.

DNA manipulation. Plasmid and chromosomal DNA purification, restriction, and electrophoresis in agarose gels were performed by using well-established protocols (3).

Northern blot analysis. Probes for specific genes were generated by PCR amplification with chromosomal DNA from strain GC4468 as the template and gene-specific primers obtained from Sigma-Genosys. Typically, PCR amplifications were done with 30 cycles of annealing at 60°C (45 s), elongation at 72°C (1 min), and denaturation at 94°C (30 s). The PCR products were resolved by electrophoresis in 1.25% agarose gels, recovered by excision from the gel, and purified with Qiaquick DNA-binding microspin columns (Qiagen). The DNA fragments were labeled by using Klenow DNA polymerase fragment, random-hexamer primers (Gibco BRL), and [^{32}P]dCTP (3,000 Ci/mmol) plus unlabeled dATP, dGTP, and dTTP. The labeled probes were purified by gel filtration in Sephadex G-25 columns (Pharmacia). For the Northern blot experiments, 2 to 5 μg of total RNA per lane was run in 1.25% agarose gels containing formaldehyde and transferred to Nytran membranes by using a Turboblotter setup (Schleicher & Schuell). The RNA was cross-linked to the membrane by UV irradiation, and the membranes were then hybridized at 65°C with radioactively labeled DNA fragments in cylindrical tubes by using QickHyb solution (Stratagene). The membranes were washed according to the instructions from the manufacturer. X-ray films were exposed to the membranes at −70°C and developed with a Fuji automatic developer. The radioactive signals were measured with an Applied Biosystems phosphorimager.

Construction of a *yggX* promoter deletion set and assays of transcriptional activity. Fragments of DNA containing the predicted *yggX* promoter region were amplified by PCR with primers that introduced convenient *Bam*HI and *Eco*RI sites in the reaction products. The PCR products were cloned as *Bam*HI-*Eco*RI fragments into the *lacZ* reporter vector pRJ800, a derivative of pRZ5202 (ColE1 replicon; Amp^r; multiple cloning site from pUC18). Typical PCR mixtures included 2.5 U of *Taq* polymerase (courtesy of K. Nuesslein) in ThermoPol buffer (New England Biolabs) plus 50 pmol of specific primers designed to introduce convenient restriction sites (Operon), 0.2 mM deoxynucleoside triphosphates (New England Biolabs), and 100 ng of chromosomal DNA from strain GC4468. The *E. coli* strain GC4468 was transformed to ampicillin resistance with the resulting *lacZ* fusion constructs. For β-galactosidase activity assays, overnight cultures were diluted in 15 ml of fresh Luria-Bertani broth in 125-ml Erlenmeyer flasks and were grown at 37°C with strong aeration (250 rpm). After 2 h (optical density at 600 nm of approximately 0.5), the cultures were exposed to 250 μM paraquat (PQ) for 30 min, and cells were assayed as described by Miller (37).

In vitro binding of SoxS by gel mobility shift assay. In vitro binding of SoxS protein to DNA targets was done as described previously (28). Essentially, purified SoxS protein was diluted in a buffer containing 10 mM sodium acetate (pH 5.0), 75 mM sodium chloride, and 1 mM dithiothreitol and incubated with end-labeled PCR fragments. Typical binding reaction mixtures contained 10 mM Tris-HCl (pH 8.0), 75 mM potassium chloride, 2 mM dithiothreitol, 10 fmol of ^{32}P-end-labeled PCR product, 1 pmol of randomized 32-mer oligonucleotide, and different amounts of purified SoxS protein as indicated, in a total volume of 20 μl. The binding reaction mixtures were incubated at 20°C for 20 min and run in 5% polyacrylamide gels (20 mM Tris-HCl [pH 8.0], 3 mM sodium acetate, and 1 mM EDTA) at 200 V for 3 to 4 h. Gels were dried and visualized by autoradiography.

Construction of a *yggX* deletion mutant. The method of Datsenko and Wanner (9) was used to construct a Δ*yggX* strain. Briefly, a PCR product containing the *kan* gene and flanked by the phage lambda Red recombinase recognition sites was obtained by using custom designed primers and plasmid pKD13 as a template. The PCR product also contained 40-bp ends with sequences identical to the regions bordering the *yggX* coding region. GC4468 cells harboring plasmid pKD46, which allows the expression of lambda Red recombinase under the control of the *araBAD* promoter, were transformed to Kan^r by using this PCR construct. Kanamycin-resistant colonies were tested for the simultaneous loss of the resident *yggX* gene and replacement by the *kan* marker by diagnostic PCR with an insert-specific primer and a gene-specific primer. The *kan* gene was excised from the *yggX* locus by transformation of the *yggX*::*kan* strain with pCP20, a plasmid that expresses the yeast Flp recombinase and recognizes sites included in the *yggX*::*kan* construct. This kanamycin-sensitive Δ*yggX* derivative strain was named PP139.

Construction of an arabinose-regulated *yggX* gene. A PCR product containing the complete *yggX* coding region plus restriction sites included in the primers was cloned into plasmid pBAD28 (23) as a *EcoR*I-*Sal*I fragment, and the resulting plasmid was named pJP133. This plasmid was tested for complementation of the PQ sensitivity phenotype of strain PP139 (GC4468 Δ*yggX*). Strain PP139/pJP133 grew very poorly in M9 medium supplemented with 0.4% glucose and 50 μM PQ but grew normally in M9 medium supplemented with 0.4% arabinose (data not shown). Strain PP139/pBAD28 grew poorly in M9 medium in the presence of 50 μM PQ either with glucose or with arabinose as a carbon source (data not shown).

Aconitase assay. Aconitase activity was measured as described by Skovran and Downs (47). Cells were grown in Luria-Bertani broth supplemented with 1 mM L-arabinose and 50 μg of ampicillin per ml when required. Overnight cultures were diluted 1/100 in 2 ml of fresh medium in 20-mm tubes and grown at 37°C with strong aeration (275 rpm). The cultures were grown to an optical density at 600 nm of ~0.5 and treated with 500 μM PQ for 1 h. After this exposure, cells were harvested, washed once with cold 20 mM Tris-citrate buffer (pH 8), and resuspended in 0.5 ml of the same buffer. The cells were lysed by sonication, and the lysates were cleared by centrifugation at 14,000 rpm in an Eppendorf 5417 microcentrifuge for 1 min in a microcentrifuge. The aconitase activity was assayed at room temperature by spectrophotometry, monitoring the increase of absorbance at 240 nm after lysates were mixed with 20 mM isocitrate. Protein concentrations were measured by the method of Bradford, and specific activities were calculated as the change in absorbance per minute per milligram of protein.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or comments	Reference
Strains		
GC4468	K-12 Δ*lacU169 rpsL*	18
DJ901	GC4468 Δ*soxRS*	19
PP139	GC4468 Δ*yggX*	This work
MB101	GC4468 Δ*robA*	6
MB102	GC4468 Δ*marA*	39
Plasmids		
pRJ800	*lacZ* promoterless fusion vector, ColE1, *bla*	42
pKD13	*oriγ kan bla*	9
pKD46	[*oriR101*] [*repA101*(Ts)] *araBp-gam-bet-exo bla*	9
pCP20	Thermosensitive replicon, yeast flp recombinase, *bla*	9
pJP115	*lacZp-soxS bla*	40
pMB101	*lacZp-robA bla*	6
pMB102	*lacZp-marA bla*	39
pBAD28	Expression vector, transcription driven by *pBAD*, *bla*	23
pJP133	*araBADp-yggX bla*	This work

RESULTS

The *yggX* and *mltC* genes are transcribed under SoxS control. In order to test the transcriptional activation of the *yggX* gene under oxidative stress, we performed Northern blotting of

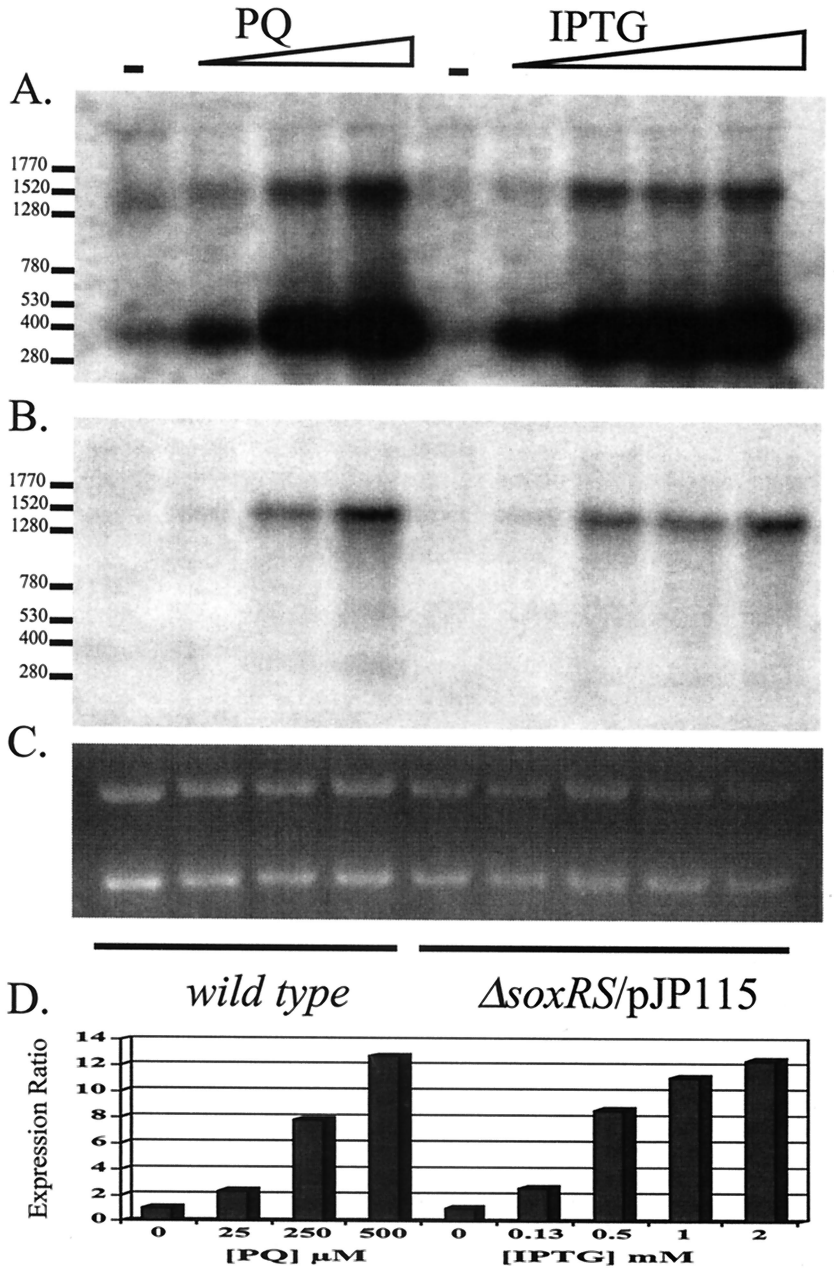

FIG. 2. Expression of *yggX* and *mltC* under oxidative stress. (A) Northern blot analysis of *yggX* transcription. A PCR fragment containing the complete coding sequence of *yggX* was radioactively labeled and hybridized with total RNAs from strain GC4468 (wild type) exposed to increasing concentrations of PQ (0, 25, 250, and 500 μM) and from strain DJ901/pJP115 exposed to increasing concentrations of IPTG (0, 0.125, 0.500, 1, and 2 mM). The positions and lengths in bases of RNA molecular size standards are indicated. (B) Northern blot analysis of *mltC* transcription. The membrane used for panel A was stripped and reprobed with a radioactively labeled PCR fragment containing the complete *mltC* coding region. The positions and lengths in bases of RNA molecular size standards are indicated. (C) Loading control. A duplicate gel was stained with ethidium bromide and visualized under UV light. (D) Quantitation of *yggX* signal. The radioactive intensity for the ~400-nt bands in the Northern blot was measured by phosphorimaging. The values shown are the relative activation ratios for the treatments, normalized to the signal intensity in the untreated sample.

mRNA isolated from *E. coli* cultures. Strain GC4468 (wild type) was exposed to increasing concentrations of PQ, a redox-cycling agent that generates intracellular superoxide in a reaction dependent on NADPH and at least three diaphorases (30, 31). Strain DJ901/pJP115 (Δ*soxRS*/pSoxS) was exposed to increasing concentrations of IPTG (isopropyl-β-D-thiogalactopyranoside), an artificial inducer of the *soxS* gene under the control of the *lacZ* promoter in pJP115 (39). A PCR product spanning the whole *yggX* coding sequence was radioactively labeled and used as a probe for *yggX* expression (Fig. 2A). The autoradiogram of this Northern blot showed two transcripts. The smaller transcript comigrated with a 400-nucleotide (nt)

molecular size marker, consistent with the size of the *yggX* coding region and the known promoter structure (15). The steady-state level of this transcript was elevated in a dose-dependent manner by either treatment with PQ or expression of SoxS. The relative abundance of this ~400-nt transcript was measured by phosphorimaging (Fig. 2D). A larger transcript comigrated with a 1,520-nt molecular size marker and was activated by either treatment with PQ or expression of SoxS.

The size of the larger transcript was consistent with either a *mutY-yggX* message starting from one of the promoters upstream of *mutY* or, alternatively, a *yggX-mltC* message starting at the promoter upstream of *yggX* (Fig. 1). To distinguish between these two possibilities, the filter from the previous experiment was stripped and reprobed with a PCR fragment spanning the complete *mltC* coding region (Fig. 2B). A single transcript was detected, and its size was estimated at ~1.5 kb. The steady-state level of this transcript containing *mltC* was increased by exposure to PQ or expression of SoxS. Due to the low levels of the basal *mltC* signal, reliable quantitation by phosphorimaging was difficult to obtain. However, we estimated that induction of *mltC* is similar in ratio to the *yggX* induction (data not shown).

In contrast, when the *mutY* coding region was used as probe, a ~1.5-kb transcript was detected, but this transcript did not show any activation by either treatment with PQ or expression of SoxS (data not shown). This lack of activation of *mutY* by PQ was consistent with the observations of Gifford et al. (14). Finally, probing the activity of the *nupG* gene with a PCR product spanning the *nupG* coding region revealed a single transcript of the expected size (~1 kb), which showed no variation with PQ treatment or SoxS expression (data not shown). These results suggest that the transcription of *yggX* and *mltC*, but not that of *mutY* or *nupG*, is activated by oxidative stress and that ectopic expression of the SoxS protein is sufficient to evoke this activation. Additionally, the *yggX* gene can be transcribed alone or as a transcript including both *yggX* and *mltC*.

Purified SoxS binds to the *yggX* promoter. The observation that SoxS regulates the *yggX-mltC* promoter was inconsistent with a previous suggestion that SoxS binding sites are absent from this regulatory region (15). To rule out an indirect effect of SoxS on the activation of *yggX*, we mapped the putative SoxS binding site by constructing a set of nested deletions of the *yggX* promoter region (Fig. 3A). These deletion fragments were fused to a *lacZ* reporter gene, and β-galactosidase activity was measured for strains carrying each construct, both untreated and in the presence of 250 μM PQ. A *lacZ* fusion containing the 223 bp upstream from the *yggX* translational start site was regulated by oxidative stress. Conversely, a fragment containing the 163 bp upstream from the *yggX* translational start site was not regulated. In vitro binding experiments with purified components showed that SoxS formed a complex with the 223-bp fragment of the *yggX* promoter (Fig. 3B). In contrast, SoxS failed to form any detectable complex with the smaller, 163-bp fragment (Fig. 3B). Sequence analysis revealed a match for an inverted SoxS binding site (5′-TAGGCACAA TATCTAAGTGG-3′) between positions 190 and 171 upstream from the *yggX* translational start site. To confirm the position of the SoxS binding site, two additional deletions of the *yggX* promoter were constructed and fused to *lacZ*. The *lacZ* fusion containing the 193 bp upstream from the *yggX* translational start site and harboring the proposed SoxS binding site was regulated by oxidative stress (Fig. 3A). Conversely, a fragment containing the 173 bp upstream from the *yggX* translational start site and lacking 17 out of the 20 bases of the putative SoxS binding site was not regulated (Fig. 3A). These in vitro binding and in vivo regulation results show that SoxS is directly involved in the transcriptional regulation of the *yggX* gene.

The SoxS paralogs MarA and Rob are not sufficient for activation of *yggX*. The SoxS protein is a member of the AraC-XylA family of transcriptional activators (12). The closest SoxS paralogs are MarA, a protein involved in multiple-antibiotic resistance (1), and Rob, an abundant protein of uncertain physiological relevance (6, 46). MarA and Rob can activate several of the SoxS-activated genes, since both MarA and Rob recognize the same DNA sequence for binding (26, 27). To test whether either MarA or Rob could activate the transcription of *yggX*, we measured the relative level of *yggX* mRNA in cells in which the expression of MarA or Rob could be selectively activated. This selective expression of MarA and Rob was achieved by using strains MB102 (Δ*marA*) (39) and MB101 (Δ*robA*) (6). Both strains are GC4468 derivatives transformed with plasmids that allow, respectively, the expression of MarA or Rob under the control of IPTG-regulated promoters. As shown before, the levels of *yggX* mRNA were increased after treatment with PQ or expression of SoxS (Fig. 4A). In contrast, the level of *yggX* transcript was unaffected by the expression of MarA or Rob (Fig. 4A). As a positive control for transcriptional activation by MarA and Rob, the membrane from the former Northern blot was stripped and rehybridized with a labeled PCR product containing the *inaA* coding region. The *inaA* gene is activated by SoxS, MarA, and Rob, with MarA being the strongest activator (34, 43). This experiment confirmed the transcriptional activity of artificially expressed MarA and Rob proteins (Fig. 4B) and verified that they do not activate *yggX* expression.

***A yggX* deletion mutant is hypersensitive to PQ.** It was reasonable to believe that the induction of *yggX* expression was indicative of a potential role for this gene in the resistance to oxidative stress. To test this putative role of *yggX*, we built strain PP139, a Δ*yggX* derivative of strain GC4468 (see Materials and Methods). Overnight cultures of both wild-type and Δ*yggX* strains were diluted into fresh medium in the presence or absence of 50 μM PQ and grown with strong aeration (Fig. 5). Both strains grew equally in the absence of PQ (Fig. 5A), but the Δ*yggX* strain grew poorly in medium containing PQ (Fig. 5B). The hypersensitivity of the Δ*yggX* strain was also measured in solid medium by using gradient plates (8), and this method was used to assay the growth of the *yggX* mutant exposed to various oxidative stress agents. This experiment showed that a Δ*yggX* strain was not only more sensitive to PQ than the isogenic wild-type strain but also more sensitive than a Δ*soxRS* strain (Table 2). Interestingly, other superoxide-producing drugs, such as menadione and phenazine methosulfate, had a small or no effect on the growth of PP139 on plates. This observation was unexpected, since both menadione and phenazine methosulfate produce substantial oxidative stress through the generation of intracellular superoxide. The organic peroxide *tert*-butyl-hydroperoxide had only a modest relative effect on the growth of PP139.

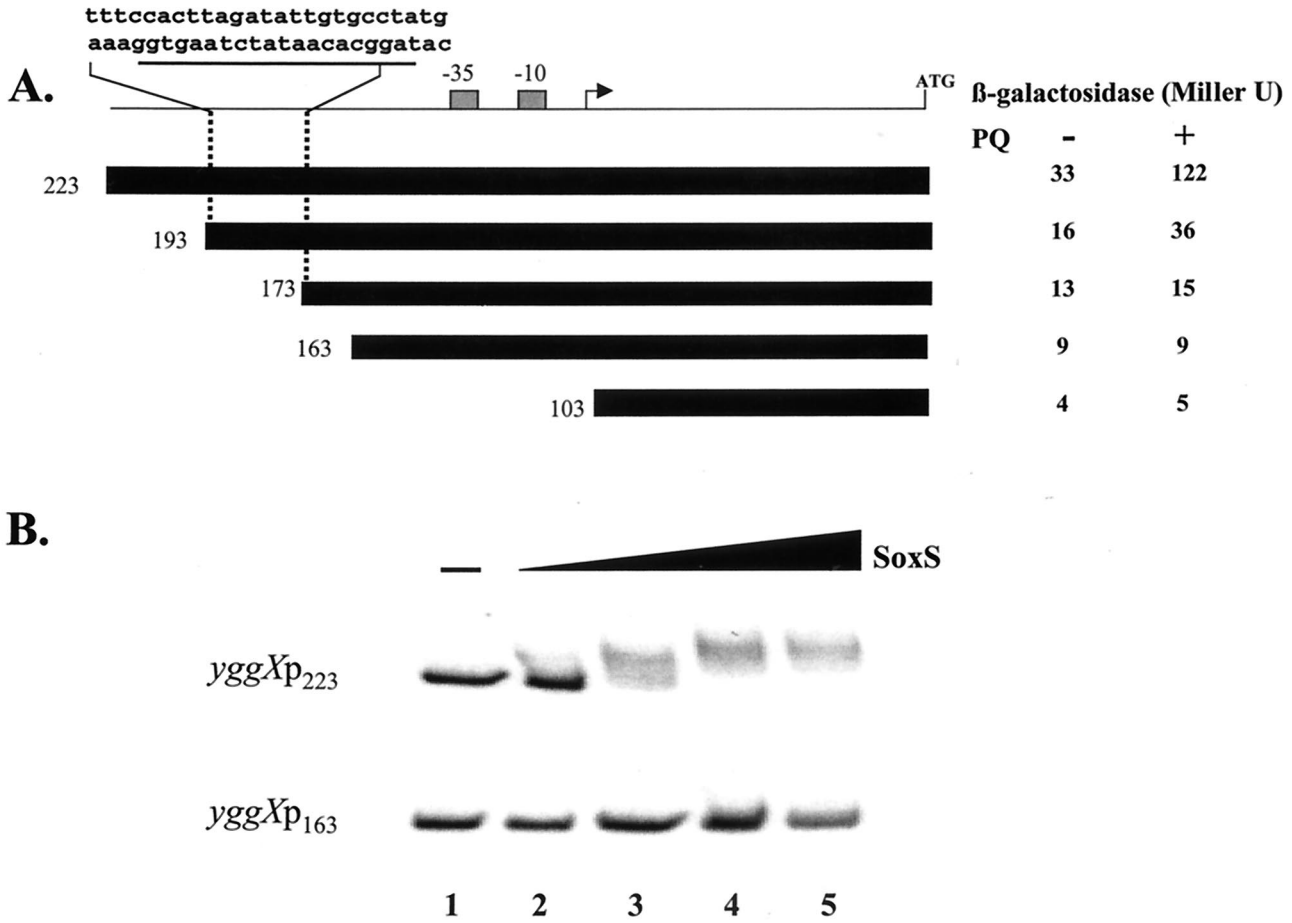

FIG. 3. A deletion set of *yggX-lacZ* transcriptional fusions. (A) Solid rectangular bars indicate the lengths of the *yggX* promoter regions fused to a *lacZ* reporter gene in pRJ800. The bracketed sequence highlights the region necessary for the activation of *yggX* transcription by PQ. The solid line shows the SoxS putative binding site. The β-galactosidase activities from strains containing each individual fusion, assayed as described in Materials and Methods, are indicated on the right; the numbers are averages of at least three independent experiments. The corresponding standard deviations were less than 15%. (B) Gel mobility shift assay of SoxS binding to the *yggX* promoter. PCR-generated fragments were incubated with increasing amounts of purified SoxS as described in Materials and Methods. Lanes 1 to 5 contained, respectively, 0, 30, 100, 300, and 1,000 ng of SoxS.

As shown before, the *yggX* and *mltC* genes are apparently cotranscribed, and therefore, the hypersensitive phenotype of a *yggX* mutant could result from polar effects on *mltC* expression. To test this possibility, we cloned the *yggX* gene under the control of the *araBAD* promoter by using the pBAD28 expression vector (23). By using the resulting plasmid, pJP133, the expression of *yggX* could be activated by the addition of arabinose. Strain PP139 (Δ*yggX*) was transformed to Amp^r with either vector pBAD28 or pJP133, grown overnight, and diluted into fresh medium supplemented with arabinose in the presence or absence of PQ. In the absence of PQ, all three strains (wild type, Δ*yggX*/pBAD28, and Δ*yggX*/pJP133) grew similarly (Fig. 5A). Alternatively, in the presence of PQ, strain PP139/pBAD28 grew poorly compared with the wild-type strain GC4468. This growth defect was complemented by plasmid pJP133, since strain PP139/pJP133 grew as well as the wild-type strain (Fig. 5B). These results show that expression of *yggX* was sufficient to complement the PQ-dependent growth defects of the Δ*yggX* strain and therefore that the hypersensitive phenotype of PP139 was not due to polar effects.

Deletion of *yggX* results in decreased aconitase activity under superoxide stress. Recent work has shown that overexpression of YggX complements the deficiency in aconitase activity displayed by *S. enterica gshA* mutants (16). This observation has been interpreted as evidence for a role of YggX in protecting or repairing oxidative damage of the Fe-S clusters that form the active site of aconitase. To test this putative protective role, we measured aconitase activity in wild-type and Δ*yggX* strains growing under oxidative stress (Fig. 6). In the absence of stress, the aconitase activities of the wild-type and Δ*yggX* strains were not significantly different, and the expression of YggX from plasmid pJP133 did not increase aconitase activity. Conversely, under oxidative stress induced by PQ, the Δ*yggX* strain showed a significant decrease in aconitase activity compared to the wild-type strain. Expression of YggX from plasmid pJP133 restored this activity and increased it to a level

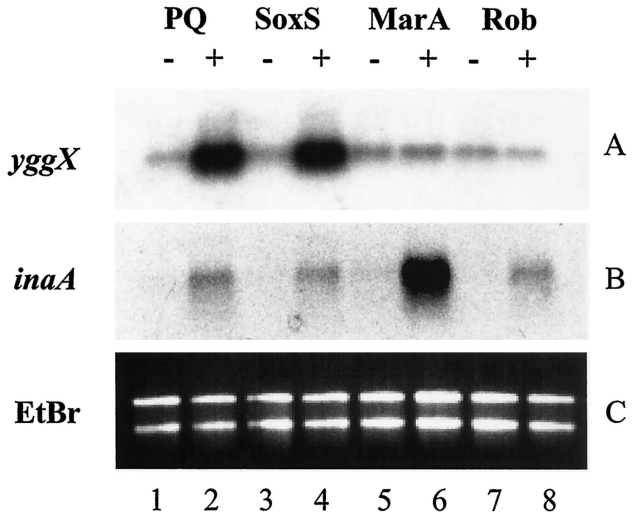

FIG. 4. Effect of MarA and Rob on the expression of *yggX*. (A) Northern blot analysis of *yggX* transcription. A PCR fragment containing the complete coding sequence of *yggX* was radioactively labeled and hybridize with total RNAs from the following strains: GC4468 (wild type) untreated or exposed to 250 μM PQ (lanes 1 and 2, respectively); DJ901/pJP115 untreated or exposed to IPTG (lanes 3 and 4, respectively); MB102/pMB102 untreated or exposed to IPTG (lanes 5 and 6, respectively); and pMB101/pMB101 untreated or exposed to IPTG (lanes 7 and 8, respectively). The concentration of IPTG was 1 mM in all cases. Only the ~400-base transcript is shown. (B) Northern blot analysis of *inaA* transcription. The filter used for panel A was stripped and reprobed with a PCR fragment containing the complete coding sequence of the *inaA* gene of *E. coli*. (C) Loading control. A duplicate gel was stained with ethidium bromide (EtBr) and visualized under UV light.

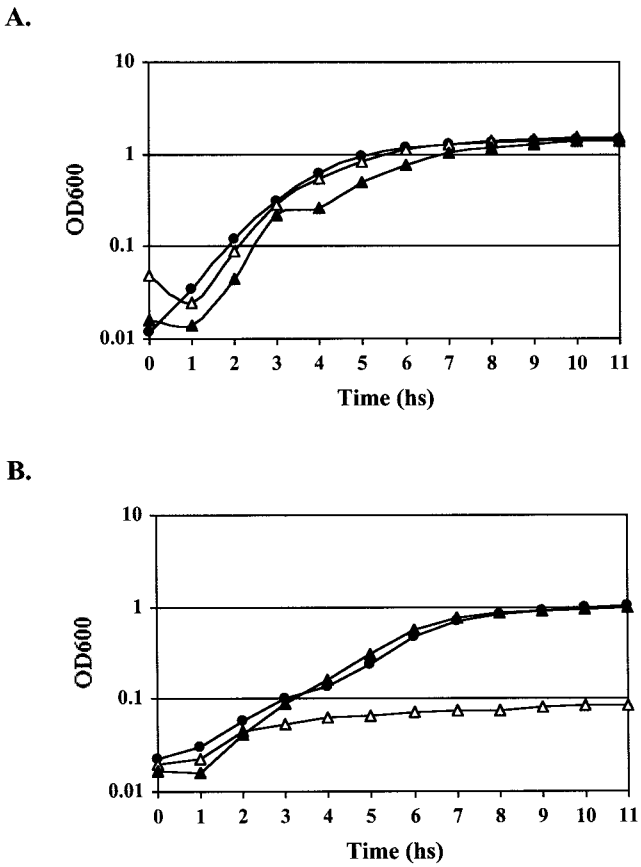

FIG. 5. PQ-sensitive phenotype of a Δ*yggX* strain. Cells were grown in M9 minimal salts medium supplemented with 0.4% arabinose, 0.1% Casamino Acids, 1 μg of thiamine per ml, 10 mM $MgCl_2$, and 1 mM $CaCl_2$. Cells were untreated (A), or PQ was added at a final concentration of 50 μM (B). Cells were grown in 125-ml Erlenmeyer flasks at 37°C and 250 rpm. Growth was monitored by measure of optical density at 600 nm (OD_{600}). Circles, GC4468 (wild type); open triangles, PP139/pBAD28 (Δ*yggX*/vector); solid triangles, PP139/pJP133 (Δ*yggX*/pYggX).

higher than that in the wild type (Fig. 6). These results showed that the product of the *yggX* gene is necessary to maintain high levels of aconitase under oxidative stress.

DISCUSSION

The *yggX* gene of *E. coli* codes for a predicted 91-residue, 11-kDa polypeptide of unknown in vivo function. Transcriptional profiling has suggested that the expression of *yggX* is activated by superoxide stress as part of the SoxRS regulon (39). The results presented here confirmed and extended the role of the SoxRS system in regulating not only *yggX* but also the downstream gene *mltC*. Our results are consistent with the genetic structure of the *mutY-yggX-mltC-nupG* region proposed by Gifford and Wallace (15) (Fig. 1). The coregulation of *yggX* and *mltC* (Fig. 2) was consistent with SoxS-mediated activation of the proximal promoter upstream of the *yggX* coding region, with a termination point between *yggX* and *mltC* that allows readthrough. The binding of purified SoxS to the *yggX* promoter strongly suggests that the PQ- and SoxS-mediated activation of *yggX* is the result of direct interaction between promoter and SoxS. A sequence necessary for PQ-mediated transcriptional induction of *yggX* was mapped to a 20-bp region between positions 173 and 193 upstream the *yggX* translational start site. This 20-bp region contained 17 bp of a partial match for a putative SoxS binding site. This putative SoxS binding sequence diverges substantially from the *mar-sox-rob* consensus (33), with only seven matches out of 14 nonrandom positions.

Additionally, and despite similarities in the structures of their DNA binding domains and cognate DNA binding sites, the SoxS homologs MarA and Rob did not activate in vivo transcription of *yggX* under the conditions tested (Fig. 4). This result was somewhat surprising, since it has been repeatedly

TABLE 2. Sensitivity to oxidants

Strain	Avg growth (cm) on gradient plates with[a]:				
	No addition	PQ	MN	PM	TBP
GC4468 (wild type)	8.0	7.5	5.1	7.6	3.2
DJ901 (Δ*soxRS*)	8.0	3.8	4.2	2.6	3.1
PP139 (Δ*yggX*)	8.0	1.3	5.1	6.3	2.5

[a] The measurements were done in duplicate and repeated at least twice with independent cultures. Values from a representative experiment are shown. The amount or maximal concentration of each agent was as follows: PQ, 2 mM; menadione (MD), 7 mg; phenazine methosulfate (PM), 1 mg; *tert*-butylhydroperoxide (TBP), 1 mM.

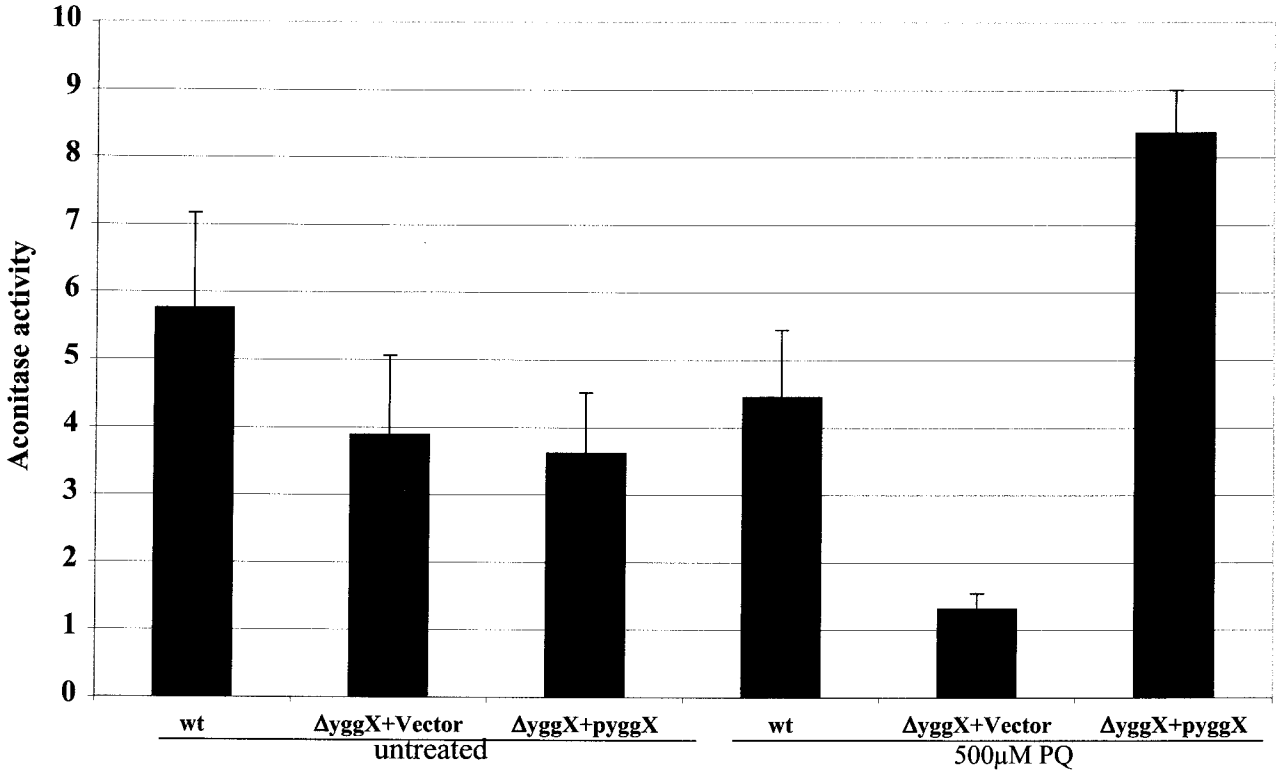

FIG. 6. Aconitase activity in wild-type (wt) and Δ*yggX* strains. Cells were grown and treated as described in Materials and Methods. Bars represent the average aconitase activities and standard deviations from three independent experiments.

shown that regulatory cross talk exists between these proteins and SoxS (1, 17, 21, 27, 35). In this context, the different degree of activation of the *inaA* gene by the three homologous proteins (Fig. 4) is noteworthy but not surprising, since such differences have been reported for other genes. For example, SoxS appears to be a better activator of *fumC* than is MarA, which is in turn more potent than Rob (2, 34). A growing body of evidence suggests that, although overlapping, the MarA, Rob, and SoxS proteins have different regulatory targets. For example, the *nfo* gene, coding for endonuclease IV, is activated by SoxS but not by MarA or Rob (17). Similarly, the *gshB* gene, coding for a glutathione synthase, is activated by MarA but not by SoxS or Rob (5, 39). Recently, Barbosa and Levy showed that the *nfnB* gene is regulated preferentially by MarA, which binds to a site that diverges substantially from the Mar-Sox-Rob consensus, with eight mismatches out of the 14 nonrandom positions (4). Hence, divergence from the Mar-Sox-Rob box consensus sequence might determine differential regulation of a promoter by these transcription factors. It is tempting to hypothesize that the sequence divergence from the consensus in the putative SoxS binding site at the *yggX* promoter and the lack of regulation by the SoxS homologs MarA and Rob are related phenomena.

Recently, a function in antioxidant defense was proposed for the *yggX* gene of *S. enterica*, as a mediator of iron transactions between uptake and Fe-S cluster synthesis and/or repair (16a). The results presented here are consistent with this hypothesis, since growth of a Δ*yggX* strain was deficient in minimal medium under oxidative stress. Expression of YggX from a plasmid was sufficient to complement this sensitivity, which shows that the phenotype was not due to polar effects. Additionally, a Δ*yggX* strain showed deficient aconitase activity under oxidative stress, while expression of YggX in that same mutant enhanced aconitase activity above wild-type levels. Interestingly, *acnA*, the gene coding for one of the two *E. coli* aconitases, aconitase A, is also a member of the SoxRS regulon. It has been proposed that the SoxS-mediated induction of aconitase A replaces the labile activity of aconitase B, helping the cell in the adaptation to oxidative stress (22). Moreover, it has been estimated that under PQ-elicited oxidative stress, more than 95% of aconitase activity is provided by aconitase A (22). We can therefore propose a model in which the transcriptional activation of both *acnA* and *yggX* results in the maintenance of high levels of aconitase activity under oxidative stress. Moreover, the level of YggX appears to be limiting for aconitase activity under PQ-induced oxidative stress, since expression of YggX from a multicopy plasmid enhances aconitase activity beyond the wild-type levels. Interestingly, a recent report showed that despite its in vivo stability, the activity of purified aconitase A is destroyed in vitro by superoxide (48). The resistance of aconitase A to oxidation was restored in vitro by addition of cell extract. The protecting agent was sensitive to boiling but not to dialysis or RNase and could not restore the activity of damaged aconitase (48). It is tempting to speculate that the soluble factor that protects aconitase A in vitro from oxidative damage is YggX. We are currently addressing this hypothesis experimentally.

The significance of the SoxS-mediated activation of *mltC* is

not clear. MltC is one of three *E. coli* lytic transglycosylases found in the outer membrane, where the enzyme modulates the turnover of the murein sacculus during cell growth (10, 11, 32). The products of murein lysis are transported into the cytoplasm and broken into peptide and disaccharide components by the amidase AmpD (25). The peptides are recycled into the cell wall as UDP-*N*-acetylmuramic acid peptide intermediates (36). The disaccharides are cleaved by the β-glucosaminidase NagZ, but the metabolic fate of the released 1,6-anhydromuramic acid is not known (20). Since *E. coli* cells under superoxide stress showed a global activation of genes involved in sugar transport (39), it is possible that the activation of *mltC* contributes to increase the intracellular sugar pools by degrading the peptidoglycan.

The possibility of stress-regulated Fe-S cluster protection or repair is certainly consistent with the present models of global antioxidant response. Furthermore, the transcriptional activation of *yggX* might not be the sole example. Recently, a study of global regulation in response to oxidative stress (52) showed the induction of genes of the *isc* region, which is known to be involved in Fe-S cluster assembly (38, 44, 45, 51). Interestingly, this transcriptional activation was independent of both OxyR and SoxRS. Therefore, stress-mediated activation of genes related to Fe-S cluster dynamics could involve multiple operons and pathways.

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