THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 275, No. 4, Issue of January 28, pp. 2505–2512, 2000
*Printed in U.S.A.*

# Thioredoxin 2 Is Involved in the Oxidative Stress Response in *Escherichia coli**

(Received for publication, September 17, 1999, and in revised form, November 4, 1999)

**Daniel Ritz‡, Hiten Patel‡, Bernard Doan§, Ming Zheng§, Fredrik Åslund‡¶, Gisela Storz§, and Jon Beckwith‡‖**

*From the ‡Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115 and the §Cell Biology and Metabolism Branch, NICHHD, National Institutes of Health, Bethesda, Maryland 20892*

**Two genes encoding thioredoxin are found on the *Escherichia coli* genome. Both of them are capable of reducing protein disulfide bonds *in vivo* and *in vitro*. The catalytic site contains a Cys-$X_1$-$X_2$-Cys motif in a so-called thioredoxin fold. Thioredoxin 2 has two additional pairs of cysteines in a non-conserved N-terminal domain. This domain does not appear to be important for the function of thioredoxin 2 in donating electrons to ribonucleotide reductase, 3′-phosphoadenylsulfate-reductase, or the periplasmic disulfide isomerase DsbC. Our results suggests that the two thioredoxins are equivalent for most of the *in vivo* functions that were tested. On the other hand, transcriptional regulation is different. The expression of *trxC* is regulated by the transcriptional activator OxyR in response to oxidative stress. Oxidized OxyR binds directly to the *trxC* promoter and induces its expression in response to elevated hydrogen peroxide levels or the disruption of one or several of the cytoplasmic redox pathways. Mutants lacking thioredoxins 1 and 2 are more resistant to high levels of hydrogen peroxide, whereas they are more sensitive to diamide, a disulfide bond-inducing agent.**

Thioredoxin is a small soluble protein capable of catalyzing thiol-disulfide redox reactions. The two cysteines of the conserved active site (W$CX_1X_2$C) form a disulfide bond after reduction of exposed disulfides in a variety of substrate proteins. The reduced form is regenerated by the flavoenzyme thioredoxin reductase which in turn is kept reduced by NADPH (1). In *Escherichia coli*, thioredoxin is an electron donor for ribonucleotide reductase, for methionine sulfoxide reductase (2), and for 3′-phosphoadenylylsulfate (PAPS)[1] reductase in the sulfate assimilation pathway (3, 4). *E. coli* thioredoxin is also an essential subunit of the DNA polymerase of bacteriophage T7 and is essential for the assembly of several filamentous phages (5). The redox activities of the protein are not required for these latter functions (6). Many additional roles for thioredoxin have been proposed in eukaryotic organisms (7, 8).

Thioredoxin can also help in the cellular defense against oxidative stresses, such as exposure to hydrogen peroxide or hydroxyl radicals (9, 10). Reactive oxygen species can oxidize cysteine or methionine residues leading to the formation of disulfide bridges or methionine sulfoxide (11). Such reactions will often inactivate cellular proteins. Reduction of both of these products may be accomplished in microbial systems by thioredoxin and thioredoxin reductase (12).

Recently, a second gene encoding a thioredoxin was discovered in *E. coli* (13, 14). *In vitro* data show that thioredoxin 2 (*trxC* gene product) is a functional thioredoxin, as it was able to reduce insulin and ribonucleotide reductases in a thioredoxin reductase-dependent manner (13) and serve as a hydrogen donor for PAPS reductase (3). These finding were confirmed *in vivo*, where the overexpressed *trxC* gene product could complement mutant strains for various functions (14). However, the wild-type expression level of thioredoxin 2 was not sufficient to replace either thioredoxin 1 or glutaredoxin 1 in the reducing pathway of sulfate assimilation, as a *trxA grxA* double mutant was not viable on minimal media (4).

Whereas thioredoxins are usually similar in size and share the three-dimensional "thioredoxin fold" structure, *E. coli* thioredoxin 2 contains, in addition, a novel N-terminal domain of 30 amino acids. This domain contains two pairs of cysteine residues both in $CX_1X_2C$ sequences. *In vitro*, this N-terminal region partly regulates the enzymatic activity of thioredoxin 2 as a protein-disulfide reductase (13). In this study, we have asked whether there are specific physiological roles attributable to thioredoxin 2 that differentiate it from thioredoxin 1, and whether the unusual N terminus of thioredoxin 2 might contribute to any such differences. Our findings indicate that there is substantial overlap in the reactions that can utilize either of the two thioredoxins. However, our studies on the regulation of TrxC suggest an important role for thioredoxin 2 in the response of *E. coli* to oxidative stress that may differentiate it from thioredoxin 1.

## EXPERIMENTAL PROCEDURES

*Bacterial Strains and Growth Conditions*—The bacterial strains and plasmids used in this study are listed in Table I. Cells were grown routinely in NZY medium (15). For testing growth on minimal medium M63 was used as described (14). To minimize background growth due to impurities, Difco agar was substituted with noble agar (Sigma) in the experiments selecting for growth of HP24. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml (plasmid) and 25 μg/ml (chromosome); chloramphenicol, 10 μg/ml; kanamycin, 30 μg/ml; and tetracycline, 15 μg/ml. D-Glucose and L-arabinose were used at 0.1% to modulate the expression of *trxC* alleles under the control of the $P_{BAD}$ promoter (16).

*Construction of trxC Mutants*—Site-specific mutations were introduced in *trxC* to change cysteine residues to serines. Wild-type *trxC* was

* This work was supported by National Institutes of Health Grant GM41883 and fellowships from the Schweizerischen Nationalfonds (to D. R.), the American Cancer Society (to M. Z.), and the European Molecular Biology Organization (to F. Å.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Present address: Dept. of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139.

‖ American Cancer Society Research Professor. To whom correspondence should be addressed: Dept. of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115. Tel.: 617-432-1920; Fax: 617-738-7664; E-mail: Jon_Beckwith@hms.harvard.edu.

[1] The abbreviations used are: PAPS, 3′-phosphoadenylylsulfate; AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid; PETG, phenylethyl thio-β-D-galactoside; PVDF, polyvinylidene difluoride; PCR, polymerase chain reaction; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

TABLE I
*Strains and plasmids used in this work*

| Strain or plasmid | Relevant genotype and features | Ref. or source |
|---|---|---|
| Plasmids | | |
| pBAD33 | Arabinose regulation, $Cm^r$ | 16 |
| pET15b | T7 regulation, $Amp^r$ | Novagen, Madison, WI |
| pNG102 | pBR322 *malF-lacZ* 10z | 35 |
| pEJS62 | pBAD33-*trxC* | 14 |
| pDR1000 | pET15b-$His_6$-*trxC* | This work |
| pDR1004 | pBAD33-*trxC* C64S/C67S | This work |
| pDR1005 | pBAD33-$His_6$-*trxC* | This work |
| pDR1006 | pBAD33-$His_6$-*Δ2–29trxC* | This work |
| pDR1007 | pBAD33-*trxC* C5S/C8S | This work |
| pDR1008 | pBAD33-*trxC* C25S/C28S | This work |
| pDR1010 | pBAD33-*trxC* C8S | This work |
| pDR1011 | pBAD33-*trxC* C8S/C25S/C28S | This work |
| pGSO105 | pUC18 *trxC* promoter region | This work |
| pFÅ20 | pBAD33-*ahpCF* | This work |
| pHMP1 | pNG102 Δ*mal trxC′-′lacZ* | This work |
| Strains | | |
| BL21(DE3) | $F^-$ *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm* (DE3) | Novagen, Madison, WI |
| A313 | *trxA*::kan *metE*::*Tn*10 | 2 |
| CAG12185 | *argE*::*Tn*10 | 36 |
| DHB4[a] | Δ(*ara-leu*)7697 *araD*139 Δ*lacX74 galE galK rpsL phoR* Δ(*phoA*)PvuII Δ*malF3 thi* | 37 |
| MC4100 | *araD*139 *relA*1 *thi rpsL*150 *flbB*5301 Δ(*lacU*139)*deoC*7 *ptsF*25 | Lab collection |
| WP813 | DHB4 Δ*trxA grxA*::kan | W. Prinz |
| FÅ369 | DHB4 *katG*::Tn10 Δ*ahpCF*::kan | 19 |
| FÅ378 | DHB4 Δ*trxA gor522* mini Tn10 | 19 |
| FÅ409 | FÅ378 *oxyR*::kan | This work |
| HP15 | DHB4 Δ(λattL-*lom*)::*bla trxC′-′lacZ* | This work |
| HP16 | DHB4 Δ*trxA trxB*::kan Δ(λattL-*lom*)::*bla trxC′-′lacZ* | This work |
| HP17 | DHB4 Δ*trxC nadB*::*Tn*10 | This work |
| HP18 | DHB4 Δ*trx* Δ(λattL-*lom*)::*bla trxC′-′lacZ* | This work |
| HP21 | FÅ378 Δ(λattL-*lom*)::*bla trxC′-′lacZ* | This work |
| HP22 | DHB4 Δ*trxA gshA20*::kan Δ(λattL-*lom*)::*bla trxC′-′lacZ* | This work |
| HP23 | DHB4 *trxB*::kan Δ(λattL-*lom*)::*bla trxC′-′lacZ* | This work |
| HP24 | WP813 Δ(λattL-*lom*)::*bla trxC′-′lacZ* | This work |
| HP27 | FÅ369 Δ(λattL-*lom*)::*bla trxC′-′lacZ* | This work |
| DR352 | Dark blue suppressor of HP24 | This work |
| DR353 | DHB4 *oxyR*::spc | This work |
| DR372 | DR352 *oxyR*::spc | This work |
| DR379 | HP24 *ahpC*::*Tn*10Cm | This work |
| DR380 | HP24 *ahpC32*::*Tn*10Cm | This work |
| DR385 | WP812 Δ(λattL-*lom*)::*bla trxC′-′lacZ* | This work |
| DR386 | HP15 *ahpCF*::kan | This work |
| DR389 | DHB4 *grxA*::kan Δ*trxC nadB*::Tn10 Δ(λattL-*lom*)::*bla trxC′-′lacZ* | This work |
| DR413 | HP15 *argE*::Tn10 | This work |
| DR414 | HP15 *oxyR* A221E *argE*::Tn10 | This work |
| DR415 | HP24 *argE*::Tn10 | This work |
| DR416 | HP24 *oxyR* A221E *argE*::Tn10 | This work |
| DR419 | DHB4 Δ*trxA* Δ*trxC nadB*::Tn10 Δ(λattL-*lom*)::*bla trxC′-′lacZ* | This work |
| GSO47 | MC4100 Δ*oxyR::kan* | 20 |

[a] A variant of DHB4 that had been cured of the F′ plasmid was used throughout this study.

amplified by PCR with pEJS62 as template and the primers fs1-*Xba*I, AAGTCTAGATACTCATCCCGAGGTTAGTT, and fs2-*Hin*dIII, CGCTAAGCTTAAAGAGATTCGTTCAGCCA. (i) To get TrxC mutants with C5S/C8S or C8S the primer fs1-*Xba*I was replaced by either GCTCTAGATACTCATCCCGAGGTTAGTTATGAATACCGT**T**A**G**TACCCAT**A**GTCAGGCCATCA or GCTCTAGATACTCATCCCGAGGTTAGTTATGAATACCGTTTGTACCCAT**A**GTCAGGCCATCA for the PCR reaction. The mutagenic base changes are shown in boldface. (ii) To get the mutations for TrxC C25S/C28S and C64S/C67S two PCR reactions were carried out. First, the 5′ end of *trxC* was amplified using pEJS62 as template and the mutagenic oligonucleotides AGTCGTGACCGC**T**GCGTCCGC**T**TTTTTGCCGCATC (C25S/C28S) or GAAATTACGG**G**AGGGGCCG**G**ACCACGGTGC (C64S/C67S) and fs1-*Xba*I as primers. The product of this PCR reaction was then used in a second round of PCR to amplify the entire *trxC* gene with pEJS62 as template and fs2-*Hin*dIII as second primer. The PCR products were cut with *Xba*I and *Hin*dIII and ligated with pBAD33 cut with *Xba*I and *Hin*dIII to give the desired plasmids. In order to get the mutant C8S/C25S/C28S pDR1008 was both digested with *Pvu*I and *Dra*III. The DNA fragment containing the 3′ part of *trxC* was then ligated with pDR1007 cut with *Pvu*I and *Dra*III. This resulted in the plasmid pDR1011.

We also constructed similar variants of TrxC to the ones described by Miranda-Vizuete *et al*. (13). The complete *trxC* coding sequence was amplified by PCR using pEJS62 as template and TAGTTATATGAATACCGTTTGTACCC as well as TTATTGCTCAGCAAGAGATTCGTTCAGCCAG as primers containing an *Nde*I and *Blp*I restriction site (underlined), respectively. The product was digested with *Nde*I and *Blp*I and ligated with the expression vector pET15b cut with the same restriction enzymes to create plasmid pDR1000. The same expression construct was also placed under the control of the arabinose promoter by cutting pDR1000 with *Xba*I and *Hin*dIII and ligating with pBAD33 cut with *Xba*I and *Hin*dIII to give pDR1005. Finally, a truncated version of *trxC* was created by PCR lacking the 5′ region coding for amino acids 2–29 again using pEJS62 as template and GTGCATATGCACGACTTGTTTGACG plus fs2-*Hin*dIII as primers. The amplified DNA fragment was cut with *Nde*I and *Hin*dIII and ligated with pDR1005 digested with the same enzymes to yield pDR1006. To obtain a *trxC* promoter plasmid, a fragment carrying the *trxC* promoter region was PCR-amplified from genomic DNA using the primers GGACAGAATTCACTTTGACC and CGGGATCCCGATTGATGGCCTGAC. The 290-base pair fragment was cloned into the *Eco*RI and *Bam*HI sites of pUC18 to generate pGSO105. The integrity of all of these plasmids was verified by DNA sequencing.

*Construction of Strains Containing a trxC′-lacZ Fusion*—To obtain a translational fusion of the N-terminal part of TrxC with β-galactosidase, a DNA fragment was amplified by PCR using chromosomal DNA from DHB4 as template and GGAATTCTCCAGTGCCAGAACGCAATC plus CCGGATCCAAGTCGATCACCACAGGTAGATCAT as primers. The first primer hybridizes to a position 814 nucleotides upstream of the predicted translation start site of *trxC* and contains an *Eco*RI site.

The second primer hybridizes within the *trxC*-coding region and contains a *Bam*HI site. The amplified DNA fragment was cut with *Eco*RI and *Bam*HI and ligated into pNG102 that had been cut with *Eco*RI and *Bam*HI as well to give plasmid pHMP1, and the integrity was confirmed by sequencing. The construct expresses a hybrid protein with the first 58 codons of TrxC fused to β-galactosidase. The fusion was then integrated into the chromosome of DHB4 using specialized transduction with lambda InCh[2] to give HP15 and subsequently transferred to other recipient strains by generalized transduction with P1 (17).

*β-Galactosidase Activity*—Assays on plate and in liquid media were performed as described (17). The β-galactosidase activity inhibitor PETG (Bachem, Torrance, CA) was added to minimal plates at a final concentration of 1 mM (18) to facilitate distinction between strains exhibiting wild-type expression levels of the *trxC′-′lacZ* fusion and mutants that had acquired a higher expression level.

*Treatment of Cell with Hydrogen Peroxide and Diamide*—To determine the effect of $H_2O_2$ and diamide (diazenedicarboxylic acid bis(*N*,*N*′-dimethylamide), Sigma) on growth rate, cells were grown to an $A_{600}$ of about 0.15 in rich medium at 37 °C, and $H_2O_2$ or diamide was then added to a final concentration of 5 mM or 800 μM, respectively. The growth of the cells was then followed by determining the absorbance at 600 nm.

*Antibodies Against Thioredoxin 2*—The plasmid pDR1000 was transformed into BL21/λDE3, and one large colony was used to inoculate 1 liter of NZ medium with 200 μg/ml ampicillin. The cells were grown to mid-log phase at 37 °C. After addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM, expression of thioredoxin 2 was then induced for 2 h. Cells were harvested and broken by three passages through a French press at 16,000 pounds/square inch. The crude extract was then applied onto a $Ni^{2+}$-affinity column (Novagen, Madison, WI). After washing the column, $His_6$-TrxC was eluted with 60 mM imidazole and dialyzed against a buffer with 20 mM Tris-HCl, pH 8.0, and stored at 1.5 mg/ml. Polyclonal antibodies against thioredoxin 2 were then raised in New Zealand White rabbits (Covance, Denver, PA). The antibodies were affinity purified on PVDF blots. Purified $His_6$-TrxC was electrophoresed on a 15% polyacrylamide-SDS gel and transferred to Immobilon P membrane (Millipore, Bedford, MA) by semi-dry electroblotting (Bio-Rad). Anti-TrxC serum was diluted 10-fold with phosphate-buffered saline, and 500-μl aliquots were incubated overnight at 4 °C with a 5 × 13-mm piece of PVDF membrane containing 60 μg of thioredoxin 2 each. After extensive washing, antibodies were eluted first with 100 μl of 0.1 M glycine, pH 2.5, and then with 100 μl of triethylamine, pH 11.7. The pH was immediately neutralized by adding 1/10 volume of 1 M Tris-HCl, pH 8.0. Glycerol was finally added to a final concentration of 20%, and the purified antibodies were stored at −20 °C.

*Determination of TrxC and DsbC Redox Status*—The *in vivo* redox state of TrxC was measured by acid trapping of thiols and alkylation with the high molecular mass molecule AMS (4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid, Molecular Probes, Eugene, OR) essentially as described (19). Aliquots of the cell cultures at mid-log phase were mixed with 1/10 volume of 100% trichloroacetic acid. Precipitated proteins were collected by centrifugation and washed once with 1 ml of acetone. The dried pellets were resuspended in 20 μl of 2% SDS, 50 mM Tris-HCl, pH 8.0, 15 mM AMS and incubated 1 h at room temperature. Finally, 12-μl aliquots of the alkylated proteins were separated on 15% polyacrylamide-SDS gels, transferred to PVDF membranes with a semi-dry blotter, and probed with anti-TrxC or anti-DsbC antibodies. The antibodies were detected by using the enhanced chemiluminescence Western blot detection kit (Amersham Pharmacia Biotech).

*Primer Extension Assay*—RNA was isolated from MC4100 and GSO47 as described previously (20). By using an oligonucleotide specific to *trxC* (GATTGATGGCCTGACAATGGGTAC), the RNA samples were subjected to primer extension assays as described elsewhere (21).

*DNase I Protection Assay*—The DNase I footprinting assay of OxyR binding to the *trxC* promoter, contained on the *Bam*HI-*Eco*RI fragment of pGSO105, was carried out as described previously (22).

## RESULTS

### *Comparison of the Functions of Thioredoxins 1 and 2*

The goal of this study was to determine the physiological roles that thioredoxin 2 might play. Here, we analyze the functions of the two thioredoxins *in vivo* and ask whether the N-terminal domain of thioredoxin 2 influences these activities.

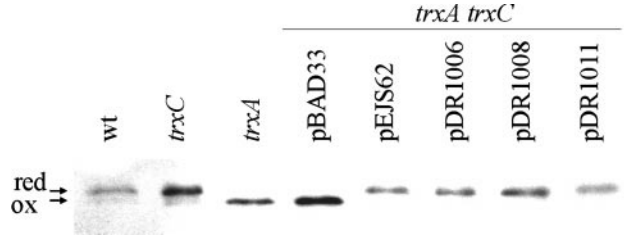


FIG. 1. **Redox state of the active-site cysteines of DsbC in various mutant backgrounds and with *trxC*-expressing plasmids.** Total cellular protein from HP15 (wild type, *wt*), HP17 (*trxC*⁻), HP18 (*trxA*⁻), and DR419 (*trxA*⁻ *trxC*⁻) containing an empty control plasmid (*pBAD33*) or expressing wild-type TrxC (*pEJS62*), TrxCΔ2–29 (*pDR1006*), TrxC C25S/C28S (*pDR1008*), or TrxC C8S/C25S/C28S (*pDR1011*) was precipitated with trichloroacetic acid, treated with AMS, and separated by SDS-polyacrylamide gel electrophoresis. DsbC was detected by Western blotting using anti-DsbC antibodies. The positions at which oxidized (*ox*) and active-site-reduced (*red*) DsbC migrate are indicated by *arrows*.

For this purpose, we have constructed derivatives of the *trxC* gene on a plasmid that expressed proteins altered either in the active site cysteines of the thioredoxin fold or in the N-terminal domain containing the additional four cysteines.

Each of the three pairs of cysteines in thioredoxin 2 were mutated to serines (the third pair being the active-site cysteines). However, we found that the mutation of cysteine residue 5 led to a significant decrease in expression (data not shown). Therefore, Cys-8 was mutated alone, and this mutation was combined with the mutation C25S/C28S to give a TrxC variant that was missing three out of four of the extra cysteines. Finally, we constructed a deletion of the N-terminal domain of TrxC (amino acids 2–29). As the expression level of the truncated TrxC is lower than the wild type, we incorporated the constructs into an expression vector in which they were fused to an N-terminal linker coding for a $His_6$ tag. In this vector, induction by arabinose of the pBAD promoter leads to expression levels of thioredoxin 2 comparable to those of thioredoxin 1.

A *trxA, grxA* double mutant is unable to grow on minimal media because of a requirement for either thioredoxin 1 or glutaredoxin 1 for PAPS reductase activity. This defect results in a cysteine requirement on minimal media. Previous results have shown that thioredoxin 2 expressed from a plasmid complements this growth defect (14). We find that the influence of the N-terminal domain on the ability to complement the growth defect on minimal medium is minimal, as all TrxC mutant variants that have an intact active site are capable of replacing thioredoxin 1/glutaredoxin 1 (data not shown).

Cytoplasmic thioredoxin 1 is also required to maintain the proper redox state of the periplasmic protein, DsbC. DsbC is itself a thioredoxin-like protein that has protein disulfide isomerase activity. The active-site cysteines are kept in a reduced state by the integral membrane protein DsbD, which in turn derives reducing power from cytoplasmic thioredoxin 1. A mutant strain lacking thioredoxin 1 (*trxA*) or thioredoxin reductase (*trxB*) accumulates oxidized DsbC suggesting that thioredoxin 1 ordinarily is the exclusive hydrogen donor to DsbD/DsbC (23). However, overexpression of thioredoxin 2 did restore the reduction of DsbC to a mutant completely lacking any thioredoxin (Fig. 1). Mutational changes in the N-terminal domain of TrxC did not alter the capacity to complement (Fig. 1). Again, although chromosomal expression of TrxC is not sufficient to maintain DsbC in a reduced state, thioredoxin 2 is able to perform this function when expressed at levels comparable to that of thioredoxin 1.

Finally, we looked at methionine sulfoxide reductase, which requires thioredoxin 1 but not thioredoxin 2 for its activity;

[2] Boyd, D., Weiss, D., Chen, J., and Beckwith, J., *J. Bacteriol*, in press.

mutations in *trxA* alone elicit a mutant phenotype (2). A methionine auxotrophic strain (*e.g. metE*) cannot grow on methionine sulfoxide as sole source of methionine if thioredoxin 1 is missing. Previous results suggested that overexpressed thioredoxin 2 can only poorly complement this defect (14). We considered the possibility that the N-terminal domain of thioredoxin 2 interferes with its ability to interact with some thioredoxin 1 substrates. However, none of the several mutant TrxC proteins were able to restore growth of the A313 strain on minimal medium with methionine sulfoxide when expressed at similar levels as chromosomal thioredoxin 1. We observed slight complementation only when high levels of wild-type TrxC were expressed (as is the case with pDR1005).

In summary, thioredoxin 2 is capable of complementing various growth defects caused by the lack of thioredoxin 1. It can function as hydrogen donor for PAPS reductase, ribonucleotide reductase (not shown), and is able to donate reducing equivalents to the periplasmic disulfide bond isomerase DsbC via the membrane protein DsbD. Mutants, which lack one or several N-terminal cysteines or have this domain entirely removed, can still function as well as wild-type thioredoxin 2 in these assays. However, methionine sulfoxide reductase appears to be acted upon much more efficiently by thioredoxin 1. Moreover, thioredoxin 2 is not able to restore a functional T7 phage infection cycle,[3] another example where the interaction between a substrate and thioredoxin is specific for thioredoxin 1.

### *TrxC Expression Is Regulated by the Transcriptional Activator OxyR in Vivo*

The results described above do not reveal any special physiological role for thioredoxin 2 nor does it appear to differ greatly in its specificity from thioredoxin 1. Therefore, we chose another approach to see if it was possible to distinguish a different function for this second thioredoxin. We considered that if we could identify genes that regulated the expression of *trxC*, these might give clues as to the conditions under which thioredoxin 2 becomes important to the cell.

A direct genetic selection for mutations that increase *trxC* expression is made possible by the finding that overexpression of *trxC* from a plasmid restores growth on minimal medium to the *trxA, grxA* double mutant WP813. Thus, mutations in a regulatory gene that expressed TrxC constitutively might also allow growth of the double mutant. To exclude mutations that would simply change the promoter region of *trxC* we integrated a translational *trxC′-′lacZ* fusion into the chromosome at the attachment site of the phage λ (*attB*) in a strain still carrying *trxC* (HP24). Mutant strains that are able to grow on minimal medium and at the same time are blue on indicator plates should contain mutations in trans-acting elements that are responsible for the expression of *trxC*. A wild-type strain containing the *trxC′-′lacZ* fusion (HP15) remains white on these plates with the β-galactosidase inhibitor PETG (see "Experimental Procedures"). We used two different approaches to generate mutants to test in our combined selection and screening plate assay. First, we used a transposon mutagenesis to select specifically for mutants due to the inactivation of a "repressing" element, and second we selected for spontaneous suppressors of the growth defect of HP24 on minimal plates that should yield a wide array of either loss of function or gain of function mutations.

*Analysis of Tn10-induced Mutants*—Transposon mutagenesis with a recombinant λ phage NK1324 that carries a mini-*Tn*10 with a chloramphenicol resistance marker on HP24 was done as described (24). 12 mutants were obtained; all of them showed a dark blue color on plates with chromogenic substrate X-gal, indicating that *trxC* was activated in these strains. By using P1 transduction, we found that only two mutants showed linkage of the chloramphenicol resistance to restoration of growth on minimal plates. We amplified DNA fragments flanking the *Tn*10 insertion site of these two mutants by PCR using chromosomal DNA as template and arbitrary primers plus a *Tn*10-specific primer (25). The PCR fragments were then sequenced to determine the transposon insertion site. In both mutants (DR379 and DR380), the transposon was inserted in the same region as follows: one insertion was 29 nucleotides upstream of the translation start site of *ahpC,* whereas the second strain had the *Tn*10 insertion in the coding region of *ahpC*, disrupting the open reading frame at codon 32. *ahpC* encodes the smaller subunit (C) of a two-subunit protein complex that has alkyl hydroperoxidase activity (26) and an active site disulfide bond. The larger subunit (F52) is highly homologous to thioredoxin reductase (27) and can directly interact with AhpC and reduce its active site cysteines in an NADH-dependent manner (28).

*Analysis of Spontaneous Suppressors of HP24*—Spontaneous suppressors arose on minimal plates at a frequency of about $10^{-7}$. All mutants that grew on the selective minimal plates plus X-gal were blue. One mutant was found that appeared to express *trxC* at a much higher level than the other suppressors, as the colonies exhibited a dark blue color. Expression of β-galactosidase from the *trxC′-′lacZ* fusion in most of the mutants gave 400–600 Miller units, whereas the deeper blue isolate (DR352) showed about a 3-fold higher activity. The *ahpC* mutant (DR379) isolated as result of the *Tn*10 mutagenesis also gave about 400 units (Table II). The majority of mutants that answered our selection and screening procedure had acquired mutations that allowed them to overexpress *trxC* 7–10-fold. This is about the degree of overexpression we know is capable of restoring growth to WP813 or HP24 when expressed from a plasmid. In order to determine whether some of our non-transposon-induced mutants affected the expression of *ahpCF,* we transformed a plasmid expressing *ahpCF* from the *araB* promoter (pFÅ20) into several mutants and assayed for complementation, the reduction of *trxC′-′lacZ* expression. DR386, an *ahpC*::kan mutant strain, showed a 5-fold lower β-galactosidase activity upon induction of the plasmid-borne *ahpCF* locus. All but one of the blue mutants that we assayed in this way (6) had the same reduction in activity, suggesting that in these strains the chromosomal *ahpC* locus was affected. On the other hand, after transformation of pFÅ20 into the suppressor DR352, the strain still retained 80% of the initial activity, suggesting that this strain carried a mutation in a different locus.

Mutations in *ahpCF* eliminate alkyl hydroperoxidase activity, resulting in higher levels of hydrogen peroxide in cells. High levels of hydrogen peroxide cause oxidative stress for the bacteria and induce a response mediated by the regulatory protein OxyR. The alkyl hydroperoxidase itself is induced by this stress response. In addition, OxyR regulates the expression of the *grxA* and *gor* genes, coding for glutaredoxin 1 and glutathione oxidoreductase, respectively (29). The glutathione, glutaredoxin pathway is involved in disulfide reductions and thiol transfers. Thus, it seemed possible that the induction of TrxC that we observed in the *ahpCF* mutants was due to activation of OxyR and that the one uncharacterized mutation in DR352 was in the *oxyR* gene.

To determine whether overexpression of TrxC in DR352 was due to a mutation in *oxyR*, we first used P1 transduction to replace the *oxyR* locus of DR352 with an *oxyR* null mutation from DR353, resulting in the mutant DR372. Eliminating

[3] E. Stewart, personal communication.

TABLE II
*β-Galactosidase activity of various strains expressing the chromosomal trxC′-′lacZ fusion*

| Strain | Relevant genotype | β-Galactosidase activity |
|---|---|---|
| HP15 | Wild-type | 60 ± 12 |
| DR353 | *oxyR* | 56 ± 16 |
| DR352 | *trxA grxA* supp | 1249 ± 262 |
| DR372 | *trxA grxA* supp *oxyR* | 49 ± 18 |
| DR413 | *argE* | 73 ± 35 |
| DR414 | supp *argE* | 1100 ± 254 |
| DR415 | *trxA grxA argE* | 95 ± 19 |
| DR416 | *trxA grxA* supp *argE* | 1873 ± 351 |
| DR379 | *trxA grxA ahpCF::cat* | 380 ± 82 |

OxyR function resulted in the abolition of the observed increase in *trxC′-′lacZ* expression (Table II). Furthermore, we used P1 transduction with a Tn*10* insertion linked to OxyR (CAG12185) to move the *oxyR* region of DR352 into other strains. The results of these transductions showed that moving this region into HP24 suppressed the growth defect of HP24 (DR416) and into HP15 (DR414) resulted in induction of the *trxC′-′lacZ* fusion, whereas transduction of the Tn*10*-linked wild-type oxyR locus did not result in any changes (DR415 and DR413, Table II). We proceeded to sequence the *oxyR* gene of DR352 and found a single C to T exchange resulting in the mutation of the conserved amino acid alanine 221 to a glutamate residue. The residue is conserved among all of the OxyR homologues (not shown), and it is located in the C-terminal region of the protein 22 residues from the active site cysteine 199. Finally, we used PCR to sequence the *oxyR* region of a number of transductants of HP15, which either did or did not show suppression of the growth defect and showed a complete correlation between the presence of the mutation and suppression of the growth defect.

Here we found that the expression of *trxC* could be stimulated in two different ways, both of them likely involving the OxyR transcription factor either indirectly by inactivation of a subunit of alkyl hydroperoxidase or directly by mutation of *oxyR*. Since OxyR can be activated either by challenge with an oxidant such as $H_2O_2$ or directly by a change in the cellular thiol-disulfide status, we determined the effect of a change in the latter state of the cell on the expression of *trxC*. The *trxC′-′lacZ* fusion was transferred by P1 transduction into various mutants with defects in one or several components of the cytoplasmic thioredoxin or glutaredoxin pathways. The steady state level of the TrxC fusion protein is about 4-fold higher in mutants where both reducing systems are affected (Table III, HP21 and HP22). Whereas the mutation in thioredoxin reductase (HP23), glutaredoxin 1 (DR385), or thioredoxin 2 (HP17) does not appear to induce any *oxyR*-dependent response on rich media, the lack of thioredoxin 1 only leads to about 2-fold increase (Table III). In comparison, the levels were about 15-fold higher in the mutant HP27 which lacks both catalase and alkyl hydroperoxidase, resulting in increased intracellular levels of peroxides ((19) Table III). The change in steady state level of *trxC* expression measured by the β-galactosidase assay was further confirmed by direct measurement of cellular thioredoxin 2 levels (Fig. 2).

TABLE III
*β-Galactosidase activity of various mutant strains deficient in components of the thioredoxin and glutaredoxin pathways expressing the chromosomal trxC′-′lacZ fusion*

| Strain | Relevant genotype | β-Galactosidase activity |
|---|---|---|
| HP18 | *trxA* | 121 ± 6 |
| HP17 | *trxC* | 61 ± 4 |
| HP23 | *trxB* | 50 ± 6 |
| DR385 | *grxA* | 66 ± 3 |
| HP16 | *trxA trxB* | 100 ± 17 |
| HP24 | *trxA grxA* | 115 ± 14 |
| DR419 | *trxA trxC* | 120 ± 14 |
| DR389 | *trxC grxA* | 49 ± 9 |
| HP21 | *trxA gor* | 263 ± 35 |
| HP22 | *trxA gshA* | 157 ± 19 |
| HP27 | *ahpCF katG* | 885 ± 6 |

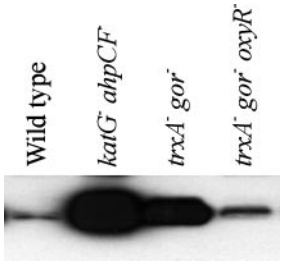


FIG. 2. **The expression of *trxC* is regulated by hydrogen peroxide and the redox state of the cell and is dependent on OxyR.** Equal amounts of total cellular protein from DHB4 (*wild type*), FÅ369 (*katG⁻ ahpC⁻*), FÅ378 (*trxA⁻ gor⁻*), and FÅ409 (*trxA⁻ gor⁻ oxyR⁻*) was separated by SDS-polyacrylamide gel electrophoresis. Thioredoxin 2 was detected by Western blotting using anti-TrxC antibodies.

### *Role of Thioredoxins during Oxidative Stress*

Because the expression of *trxC* is dependent on OxyR, we wanted to determine how thioredoxin mutants responded to oxidative stress. We found that the wild-type HP15 is sensitive to high levels of hydrogen peroxide, and growth is stopped after a 5 mM pulse of $H_2O_2$ (Fig. 3*A*). The same is true for mutants lacking thioredoxins 1 or 2 (HP18 and HP17). Surprisingly, mutants without any thioredoxin (DR419) were more resistant to $H_2O_2$ (Fig. 3*A*). These results agreed well with the finding that the surviving fraction of HP15 and HP17 cells after a 60-min exposure to 10 mM hydrogen peroxide was only about 0.3%, whereas it was significantly higher for HP18 and HP419 (25–40%). One simple explanation for this finding is that, due to the absence of thioredoxins, the equilibrium of reduced and oxidized OxyR is shifted more toward the oxidized (*i.e.* active), form and therefore the oxidative stress response including the expression of catalase and alkyl hydroperoxidase is partially activated. This activation would result in an apparent higher resistance to reactive oxygen species such as hydrogen peroxide. If the expression level of the *trxC′-′lacZ* fusion is taken as a measure of the amount of activated OxyR in the cell, then the OxyR A221E mutation should make the cells completely resistant to effects of oxidative stress by hydrogen peroxide. In fact, this is what we observed (Fig. 3*A*). This finding confirms the hypothesis put forth recently, stating that *E. coli* mutants whose cytoplasmic redox potential is more oxidizing than the wild type would be more resistant to oxidative stress (19). In another experiment we showed that overexpression of *trxC* from pEJS62 did not alter the resistance of HP15, HP18, or DR419 toward hydrogen peroxide (data not shown). Yet, it also became apparent that the resistance was at least partly due to the action of alkyl hydroperoxidase because the induction of the OxyR response was not able to rescue the mutant DR379.

Although thioredoxin activities do not seem to be necessary to confer resistance to hydrogen peroxide, they seem to contribute to the reduction of cytoplasmic disulfide bonds. We used the membrane-permeable thiol-specific oxidizing agent diamide, which temporarily inhibits growth of *E. coli* when added to growing cells. The lag phase is proportional to the amount of diamide added, and there is evidence that it depends on the capacity of the cells to reduce cytoplasmic disulfides (30). We used higher concentrations of diamide than previously de-

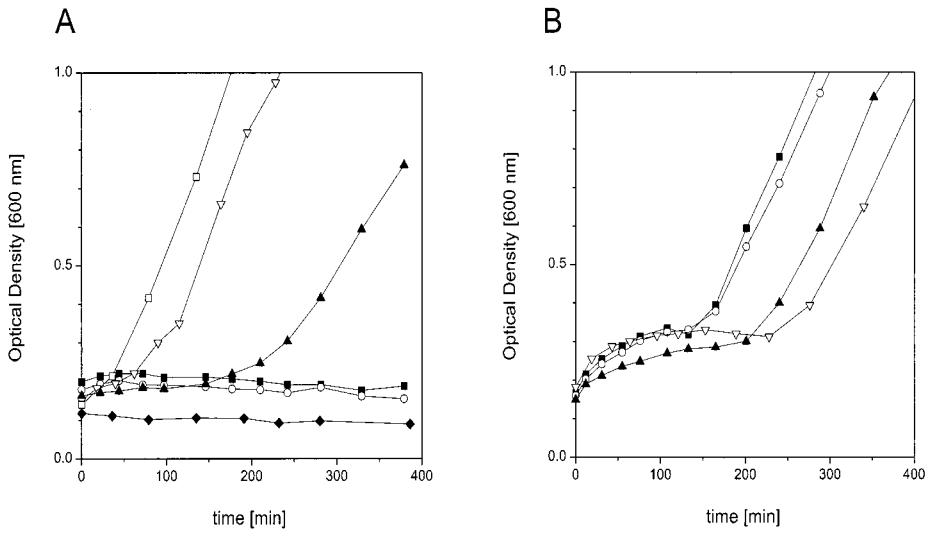


FIG. 3. **Effect of hydrogen peroxide (*A*) and diamide (*B*) on the growth of mutants missing thioredoxins or containing mutations resulting in an OxyR response.** HP15 (wild type, ■), HP17 (*trxC*$^-$, ○), HP18 (*trxA*$^-$, ▲), DR419 (*trxA trxC*$^-$, ▽) DR414 (*oxyR*221E, □), and DR379 (*ahpC*$^-$, ◆) were grown to an $A_{600}$ of about 0.15. *A,* $H_2O_2$ was added to a final concentration of 5 mM. *B,* diamide was added to a final concentration of 800 μM.

scribed, and we found that even the growth of the wild-type HP15 was inhibited (Fig. 3*B*). Nevertheless, we observed that mutants lacking thioredoxin 1 (HP18) or even more so thioredoxin 1 and 2 (DR419) had a longer phase of growth arrest, suggesting an active role of thioredoxins in dealing with disulfide stress.

### *OxyR-dependent Peroxide Induction of trxC*

The genetic analysis strongly suggested that OxyR regulates *trxC* expression. To determine whether expression of the *trxC* mRNA is induced by hydrogen peroxide in an OxyR-dependent fashion, we carried out primer extension assays. Exponential phase cultures of wild-type (MC4100) and Δ*oxyR*::kan (GSO47) mutant cells grown in LB-rich medium were split, and half of the culture was treated with 1 mM hydrogen peroxide. After 10 min, total RNA was isolated, and the RNA was subjected to primer extension analysis using a *trxC*-specific oligonucleotide. As shown in Fig. 4*A*, the levels of the *trxC* mRNA were increased by hydrogen peroxide in the wild-type but not the Δ*oxyR::kan* mutant strain, confirming that *trxC* is part of the OxyR regulon. The primer extension assay also allowed us to map the start of the *trxC* transcript to an A residue 60 base pairs upstream of the ATG start codon.

### *OxyR Binds to the trxC Promoter*

To test for OxyR binding to the *trxC* promoter region, we carried out DNase I footprinting experiments. A 290-base pair fragment encompassing the promoter was incubated with various concentrations of purified OxyR and then subjected to DNase I digestion. The oxidized wild-type OxyR protein clearly protected a region located immediately upstream of the −35 sequence of the *trxC* promoter from digestion (Fig. 4*B*). In contrast, the $OxyR_{C199S}$ protein that is locked in the reduced conformation showed significantly lower binding affinity (data not shown). Other OxyR-activated promoters are also bound by the oxidized but not reduced protein (22). A close look at the *trxC*-binding site revealed that 12 out of 20 bases matched the proposed OxyR consensus motif (22).

### *In Vivo Redox State of TrxC*

The involvement of TrxC in an oxidative stress response raised an interesting question concerning the redox state of the non-active site cysteines in the N-terminal domain of TrxC. There are several examples where the activity of a protein is regulated by the redox environment through the reversible formation of disulfide bonds. For instance, OxyR is activated by the transient formation of a disulfide bond. The recently discovered chaperone Hsp33 is also activated by formation of disulfide bonds (31). In both cases, the reduced protein is catalytically inactive.

The biochemical analysis of TrxC suggested some sort of regulation of activity. Thioredoxin 2 was more active in the insulin reduction assay when it had been treated with a reducing agent prior to the reaction (13). We therefore wanted to know the redox state of TrxC *in vivo*. By using the AMS alkylation method, we examined the steady state situation in several mutant backgrounds. All 6 cysteines of thioredoxin 2 are completely reduced in the wild type, and this remained the case as long as thioredoxin reductase, its specific reductant, is present. A shift to a more oxidizing environment as is found in HP21 does not change the redox state of TrxC at all and neither does the elevated level of hydrogen peroxide in HP27 (not shown).

## DISCUSSION

Multiple thioredoxins are found in many cells. For example, some plant cells have been found to have as many as 12 thioredoxins (32). In *E. coli*, if one were to simply search the genome sequence, one would find 10 open reading frames that would code for thioredoxin homologues or larger proteins that contain within them thioredoxin domains (33). (This inventory excludes the glutaredoxins which are structural but not sequence homologues of thioredoxin.) Of these 10 open reading frames, only 3 correspond to cytoplasmic thioredoxin molecules, and one of these appears not to be expressed at least under aerobic conditions (34). The motivation for our current study is the presumption that *E. coli* has evolved to express two thioredoxins aerobically because they fulfill different functions, at least under some environmental/ecological condition. An alternative hypothesis is that there are advantages related to issues of gene expression that favor the evolution of more than one thioredoxin gene, one of them perhaps acting as a backup, being called upon when extra stresses are put on the cell. According to this hypothesis, the two thioredoxins carry out the same tasks. On the other hand, the fact that thioredoxin 2 contains an extra N-terminal domain with two $CX_1X_2C$ sequences tempts one to consider a different physiological role for thioredoxin 2.

We have examined a number of possible functions for thioredoxin 2 that might differentiate it from thioredoxin 1. When expressed at comparable levels, both thioredoxins are "effective" *in vivo* in the reductive reactions with PAPS reductase and the DsbD membrane protein. The additional N-terminal tail of thioredoxin 2 does not appear to play any role in its function (or lack of function) in the redox pathways we have

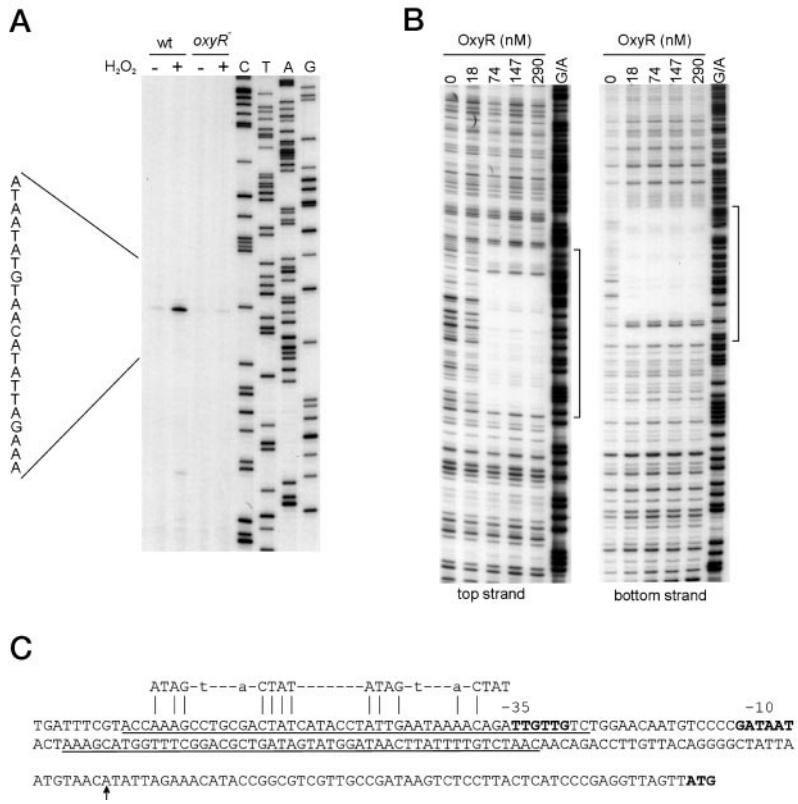


FIG. 4. **OxyR-dependent induction of *trxC* expression by peroxide stress.** *A,* primer extension assays of *trxC* expression in wild-type (*wt*) and Δ*oxyR::kan* strains grown in LB medium. Exponential phase cultures were split into 2 aliquots; 1 aliquot was left untreated, and a 2nd aliquot was treated with 1 mM hydrogen peroxide. The cells were harvested after 10 min, and total RNA was isolated. Primer extension assays were carried out with a primer specific to *trxC*. The neighboring sequencing reaction was performed using the same primer and pGSO105 as the template. *B,* DNase I footprinting assay of OxyR binding to the *trxC* promoter. The protected regions on both strands are indicated by the *brackets*. The *Bam*HI-*Eco*RI fragment of pGSO105 was labeled with $^{32}P$ at either the *Bam*HI site (*top strand*) or the *Eco*RI site (*bottom strand*), and the DNase I footprinting was carried out as described elsewhere (22). The samples were run in parallel with Maxam-Gilbert G/A sequencing ladders. *C,* similarity of the OxyR-binding site in the *trxC* promoter to the proposed consensus sequence for OxyR binding. The region protected by OxyR in both strands is *underlined*.

examined. We also found that these cysteines are maintained in a reduced state by a mechanism that does not involve any of the cytoplasmic thiol-reducing pathways. One can only speculate why the N-terminal cysteines are not susceptible to oxidation. Possibly they are involved in coordination of a metal ion as proposed (13).

To seek further a special role for thioredoxin 2, we asked whether this protein was subject to any genetic controls. By using a genetic selection for mutants with increased *trxC* expression, we have found mutations that either directly or indirectly affect the OxyR regulatory system. This indication of a role for OxyR in *trxC* expression is further supported by the finding that hydrogen peroxide increases the amount of thioredoxin 2 in cells 20-fold. Under these conditions, thioredoxin 2 appears to be more abundant than thioredoxin 1. Finally, we have shown that OxyR binds to the *trxC* promoter region via a DNA sequence that corresponds to a consensus sequence for OxyR binding.

Our results on *trxC* regulation suggest that thioredoxin 2 may play a specific role in the response to oxidative stress that differentiates it from thioredoxin 1. We have attempted to demonstrate this by seeking different properties of *trxC* mutants under conditions of hydrogen peroxide or diamide stress. In particular, we have looked at survival and at growth rates, without observing any differences from the wild type. It may be that the presence of high levels of thioredoxin 2 confers a growth or survival advantage not discernible by our tests. Such advantages may occur under specific types of oxidative stress not measured by our laboratory conditions. An alternative explanation for our findings is that *E. coli* has evolved to increase the overall concentrations of thioredoxins in the cytoplasm when confronting oxidative stress. The *trxC* gene has evolved to respond to this regulatory system because of its normally low expression levels, whereas the *trxA* gene is so highly expressed already that further increases are not easily accomplished. Moreover, as both thioredoxins are transiently oxidized after a hydrogen peroxide pulse,[4] the *de novo* synthesis of thioredoxin 2 may be an additional mechanism by which the cell tries to alleviate the consequences of oxidized proteins in the cytoplasm. According to this hypothesis, the two thioredoxins may not differ significantly in their functions and substrate specificities.

*Acknowledgments*—We thank Antonio Miranda-Vizuete for the TrxC antiserum, Bill Gillette for the oxyR mutant, Eric J. Stewart for sharing unpublished results, and Laurent Debarbieux and Hongping Tian for helpful discussions.

REFERENCES

1. Holmgren, A. (1985) *Annu. Rev. Biochem.* **54,** 237–271
2. Russel, M., and Model, P. (1986) *J. Biol. Chem.* **261,** 14997–5005
3. Lillig, C. H., Prior, A., Schwenn, J. D., Åslund, F., Ritz, D., Vlamis-Gardikas, A., and Holmgren, A. (1999) *J. Biol. Chem.* **274,** 7695–7698
4. Russel, M., Model, P., and Holmgren, A. (1990) *J. Bacteriol.* **172,** 1923–1929
5. Russel, M., and Model, P. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 29–33
6. Huber, H. E., Russel, M., Model, P., and Richardson, C. C. (1986) *J. Biol. Chem.* **261,** 15006–15012
7. Schulze-Osthoff, K., Schenk, H., and Droge, W. (1995) *Methods Enzymol.* **252,** 253–264
8. Iwata, S., Hori, T., Sato, N., Hirota, K., Sasada, T., Mitsui, A., Hirakawa, T., and Yodoi, J. (1997) *J. Immunol.* **158,** 3108–3117
9. Spector, A., Yan, G. Z., Huang, R. R., McDermott, M. J., Gascoyne, P. R., and Pigiet, V. (1988) *J. Biol. Chem.* **263,** 4984–4990
10. Takemoto, T., Zhang, Q. M., and Yonei, S. (1998) *Free Radical Biol. & Med.* **24,** 556–562
11. Moskovitz, J., Rahman, M. A., Strassman, J., Yancey, S. O., Kushner, S. R., Brot, N., and Weissbach, H. (1995) *J. Bacteriol.* **177,** 502–507
12. Farr, S. B., and Kogoma, T. (1991) *Microbiol. Rev.* **55,** 561–585
13. Miranda-Vizuete, A., Damdimopoulos, A. E., Gustafsson, J., and Spyrou, G. (1997) *J. Biol. Chem.* **272,** 30841–30847
14. Stewart, E. J., Åslund, F., and Beckwith, J. (1998) *EMBO J.* **17,** 5543–5550
15. Guzman, L. M., Barondess, J. J., and Beckwith, J. (1992) *J. Bacteriol.* **174,** 7716–7728
16. Guzman, L. M., Belin, D., Carson, M. J., and Beckwith, J. (1995) *J. Bacteriol.* **177,** 4121–4130
17. Miller, J. H. (1992) *A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
18. Schatz, P. J., Riggs, P. D., Jacq, A., Fath, M. J., and Beckwith, J. (1989) *Genes*

[4] D. Ritz, F. Åslund, and J. Beckwith, unpublished results.

*Dev.* **3,** 1035–1044
19. Åslund, F., Zheng, M., Beckwith, J., and Storz, G. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 6161–6165
20. Zheng, M., Doan, B., Schneider, T. D., and Storz, G. (1999) *J. Bacteriol.* **181,** 4639–4643
21. Storz, G., and Altuvia, S. (1994) *Methods Enzymol.* **234,** 217–223
22. Toledano, M. B., Kullik, I., Trinh, F., Baird, P. T., Schneider, T. D., and Storz, G. (1994) *Cell* **78,** 897–909
23. Rietsch, A., Bessette, P., Georgiou, G., and Beckwith, J. (1997) *J. Bacteriol.* **179,** 6602–6608
24. Kleckner, N., Bender, J., and Gottesman, S. (1991) *Methods Enzymol.* **204,** 139–180
25. O'Toole, G. A., and Kolter, R. (1998) *Mol. Microbiol.* **28,** 449–461
26. Storz, G., Jacobson, F. S., Tartaglia, L. A., Morgan, R. W., Silveira, L. A., and Ames, B. N. (1989) *J. Bacteriol.* **171,** 2049–2055
27. Tartaglia, L. A., Storz, G., Brodsky, M. H., Lai, A., and Ames, B. N. (1990) *J. Biol. Chem.* **265,** 10535–10540
28. Poole, L. B. (1996) *Biochemistry* **35,** 65–75
29. Zheng, M., Åslund, F., and Storz, G. (1998) *Science* **279,** 1718–1721
30. Prinz, W. A., Åslund, F., Holmgren, A., and Beckwith, J. (1997) *J. Biol. Chem.* **272,** 15661–15667
31. Jakob, U., Muse, W., Eser, M., and Bardwell, J. C. (1999) *Cell* **96,** 341–352
32. Mouaheb, N., Thomas, D., Verdoucq, L., Monfort, P., and Meyer, Y. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 3312–3317
33. Åslund, F., and Beckwith, J. (1999) *J. Bacteriol.* **181,** 1375–1379
34. Fetrow, J. S., Godzik, A., and Skolnick, J. (1998) *J. Mol. Biol.* **282,** 703–711
35. Froshauer, S., Green, G. N., Boyd, D., McGovern, K., and Beckwith, J. (1988) *J. Mol. Biol.* **200,** 501–511
36. Singer, M., Baker, T. A., Schnitzler, G., Deischel, S. M., Goel, M., Dove, W., Jaacks, K. J., Grossman, A. D., Erickson, J. W., and Gross, C. A. (1989) *Microbiol. Rev.* **53,** 1–24
37. Boyd, D., Manoil, C., and Beckwith, J. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 8525–8529