

Thioredoxin Is an Essential Protein Induced by Multiple Stresses in *Bacillus subtilis*

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Thioredoxin, a small, ubiquitous protein which participates in redox reactions through the reversible oxidation of its active center dithiol to a disulfide, is an essential protein in *Bacillus subtilis*. A variety of stresses, including heat or salt stress or ethanol treatment, strongly enhanced the synthesis of thioredoxin in *B. subtilis*. The stress induction of the monocistronic *trxA* gene encoding thioredoxin occurs at two promoters. The general stress sigma factor, σ^B , was required for the initiation of transcription at the upstream site, S_B , and the promoter preceding the downstream start site, S_A , was presumably recognized by the vegetative sigma factor, σ^A . In contrast to the heat-inducible, σ^A -dependent promoters preceding the chaperone-encoding operons *groESL* and *dnaK*, no CIRCE (for controlling inverted repeat of chaperone expression) was present in the vicinity of the start site, S_A . The induction patterns of the promoters differed, with the upstream promoter displaying the typical stress induction of σ^B -dependent promoters. Transcription initiating at S_A , but not at S_B , was also induced after treatment with hydrogen peroxide or puromycin. Such a double control of stress induction at two different promoters seems to be typical of a subgroup of class III heat shock genes of *B. subtilis*, like *clpC*, and it either allows the cells to raise the level of the antioxidant thioredoxin after oxidative stress or allows stressed cells to accumulate thioredoxin. These increased levels of thioredoxin might help stressed *B. subtilis* cells to maintain the native and reduced state of cellular proteins.

Thioredoxins are small, heat-stable, ubiquitous proteins with a conserved pair of vicinal cysteines (-Trp-Cys-Gly-Pro-Cys-Lys-) that undergo reversible oxidation and reduction and are efficient reductants of disulfides in low-molecular-weight compounds and proteins (23). Oxidized thioredoxins are reduced at the expense of NADPH, a reaction catalyzed by thioredoxin reductase (23). Thioredoxin systems serve as hydrogen donors, for example, for ribonucleotide reductase, phosphoadenosyl phosphosulfate reductase, and methionine sulfoxide reductase (22). In *Escherichia coli*, thioredoxin is necessary for the assembly of filamentous phages (45) and the replication of T7 (31) but is not essential for DNA synthesis and growth (21, 34).

Furthermore, thioredoxins have been implicated in the thiol-disulfide exchange and disulfide bond formation (29, 41), which are also catalyzed by glutaredoxin or protein disulfide isomerase in the endoplasmic reticula of eukaryotes and by the Dsb proteins in the periplasmic spaces of gram-negative bacteria. Protein disulfide isomerase and DsbA have been shown to assist in the folding pathway of disulfide-containing proteins both in vitro and in vivo (39).

Thioredoxin is also believed to be involved in defense against oxidative stress through its ability to reduce hydrogen peroxide (49), by acting as a hydrogen donor for a *Saccharomyces cerevisiae* peroxidase (7), or by reactivation of proteins damaged by oxidative stress or other stresses which generate reactive oxygen species (14).

We are interested in the general stress response of *Bacillus subtilis* (18). In the course of cloning and characterization of heat-inducible promoters (52), we also sequenced the regulatory region of *trxA*, the coding region of which had already been cloned and sequenced by Chen et al. (8). We investigated the expression of *trxA* and report that *trxA* encodes an essential protein, which is induced by different stress conditions, including heat and salt stress or treatment with ethanol, hydrogen peroxide, or puromycin. Two different promoters, P_B and P_A , direct the expression of *trxA*, and the stress sigma factor, σ^B , of *B. subtilis* is involved in the induction of *trxA* by stress.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All bacterial strains and plasmids used in this study are listed in Table 1. The *B. subtilis* strains were routinely grown with vigorous agitation at 37°C in synthetic medium (50) or in complex medium. The bacteria were exposed to heat, ethanol, salt, H_2O_2 , paraquat, cumene hydroperoxide (CHP), and puromycin according to a protocol described earlier (12, 44, 53). For the inhibition of the initiation of transcription, rifampin was added to a final concentration of 0.1 mg per ml.

The *E. coli* strains DH5 α and RR1 were used for DNA manipulations.

Cloning and sequencing of the regulatory region of *trxA*. Chromosomal DNA from *B. subtilis* IS58, isolated according to the method of Meade et al. (33), was digested with *Sau3AI* and cloned into the *Bam*HI-digested promoter probe vector pWH703 (52) in front of the promoterless genes coding for catechol-2,3-oxygenase (*xylE*) and chloramphenicol acetyltransferase (*cat*). After transformation of protoplasts of *B. subtilis* BD224 with the ligation mixture, clones containing promoters were isolated by selection on agar plates containing kanamycin and chloramphenicol. Catechol-2,3-oxygenase-positive clones displayed a yellow color after the colonies were sprayed with catechol due to the formation of hydroxymuconic acid semialdehyde (52). Both strands of the DNA were sequenced by the dideoxy chain termination method of Sanger et al. (47) with the primers P1 (5'-CGGCACGTGACCGCGGC-3') and P2 (5'-CCTTGTCTACA AACCC-3').

The DNA upstream of the promoter fragment inserted into pWH262 was cloned by inverse PCR. Chromosomal DNA from *B. subtilis* was digested with the restriction endonucleases *SlyI*, *EcoRI*, *PvuII*, and *ClaI*, known to cut within the coding region of *trxA*. Purified DNA fragments of the appropriate size were ligated under conditions that favor the formation of monomeric circles. Aliquots of the ligation mixture were used for PCR with the primers PtxIPCR1 (5'-TT CGTTCACGCTATTTTAATGC-3') and PtxIPCR2 (5'-TCATCATTTCACAT

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
<i>E. coli</i>		
DH5 α	F ⁻ Φ 80d Δ lacZ Δ M15 Δ (lacZYA-argF)U169 deoR recA1 endA1 hsdR17 (r _K ⁻ m _K ⁺) supE44 thi-1 gyrA69	6
RR1	F ⁻ mcrB mrr hsdS20 (r _B ⁻ m _B ⁻) ara14 proA2 lacY1 leu galK2 rpsL20 Sm ^r xyl5 mtl1 supE44	46
<i>B. subtilis</i>		
IS58	trpC2 lys-3	48
BGH1	trpC2 lys-3 sigB:: Δ (HindIII-EcoRV)::cat	32
BSA115	trpC2 rsbU::kan P _B Δ 28::P _{SPAC} rsbW313 pTet-I SP β etc::lacZ	54
BIG1	trpC2 lys-3 trxA::pHV501	pHVES1 \rightarrow IS58
BD224	trpC2 recE4	10
Plasmids		
pBluescript II SK(+/-) or KS(+/-)	Cloning vector, Ap ^r	Stratagene
pWH703	Promoter probe vector, XylE ⁺ Cm ^r Km ^r	52
pWH262	pWH703 containing a 120-bp <i>Sau</i> III A fragment carrying the 5' region of <i>trxA</i>	This study
pHV501	Integrative plasmid, Ap ^r Em ^r	51
pHVES1	pHV501 containing a 240-bp <i>trxA</i> fragment generated by PCR	This study
pKSES1	pBluescript II KS containing a 459-bp fragment generated by PCR	This study
pSKES1	pBluescript II SK containing a 720-bp <i>Cla</i> I fragment generated by inverse PCR	This study
pSKES2	pBluescript II SK containing a 950-bp <i>Eco</i> RI fragment generated by inverse PCR	This study
pSKES3	pBluescript II SK containing a 1.4-kb <i>Sly</i> I fragment generated by inverse PCR	This study
pSKES4	pBluescript II SK containing a 1.6-kb <i>Pvu</i> II fragment generated by inverse PCR	This study

TGGAGG-3'), and the PCR products were cloned into the vector pBluescript II SK(+) digested with *Eco*RV, yielding plasmids pKSES1, pSKES2, pSKES3, and pSKES4 (Fig. 1 and Table 1). Both strands were sequenced by the dideoxy chain termination method of Sanger et al. (47).

Analysis of transcription. Total RNA of the *B. subtilis* strains BD224 (carrying the plasmid pWH262), IS58, and BGH1 was isolated from exponentially growing or stressed cells by the acid phenol method described by Majumdar et al. (30) with some modifications described previously (53). Serial dilutions of total RNA were transferred onto a positively charged nylon membrane by slot blotting and hybridized with digoxigenin-labeled probes (Boehringer Mannheim) according to the manufacturer's instructions. Chemiluminograms were quantified with a Personal Densitometer from Molecular Dynamics, and induction ratios were calculated by setting the value of the control to 1. The amount of *xylE* mRNA from *B. subtilis* BD224 carrying the plasmid pWH262 was determined by slot blot hybridization with a digoxigenin-labeled 1.4-kb *Pst*I fragment, containing the *xylE* gene, from the plasmid pWH703 (52).

For the detection of *trxA* mRNA in *B. subtilis* IS58, a digoxigenin-labeled riboprobe was used. For the generation of the probe, the *trxA* gene with the potential regulatory region and the putative terminator was amplified by PCR with the primers Ptrx1 (5'-AAGCATTAATAATAGCGTGAACG-3') and Ptrx2 (5'-TGGTTCACAATTGGCGAATA-3'). The 459-bp PCR product was blunt end ligated with the vector pBluescript II KS(+), and the orientation of the cloned fragment was verified by sequencing. The resulting plasmid, pKSES1, was linearized with *Bam*HI and used as a template for in vitro transcription with T3 RNA polymerase. RNA synthesized from the coding strand after linearization of pKSES1 with *Pst*I served as a negative control for the hybridization and did not yield any specific hybridization signal (data not shown).

Northern blot analysis was carried out as described earlier (56). The primer extension analysis was performed with synthetic oligonucleotides labeled at the 5' end with [γ -³²P]ATP and complementary to the N-terminal region of *trxA* (PtrxPE1 [5'-CAAGGTCCGCACCAAGGAGC-3'] and PtrxPE2 [5'-ATTTTTCCTGATCACAGCCGG-3']) and a region preceding the *xylE* gene (PxylPE [5'-CGGCACGTGACCGCGG-3']).

Construction of a conditional *trxA* mutant of *B. subtilis*. To create a conditional mutation, a 240-bp *Hind*III-*Bam*HI-clamped fragment containing the ribosome binding site and the N-terminal part of *trxA* was amplified by PCR with the primers Ptrx3 (5'-AAGAAGCTTCATCATTCACATTGGAGG-3') and Ptrx4 (5'-GGAGGATCCTTTAACAACAAGAAGATCGG-3') and ligated with the *Hind*III-*Bam*HI-digested integration vector pHV501, generating the plasmid pHVES1. Upon transformation into *B. subtilis* IS58, pHVES1 should integrate into the chromosome via a Campbell-type integration, disrupt the *trxA* gene, and place a second copy of *trxA* under the control of the IPTG-inducible promoter P_{SPAC} (Fig. 1C). Erythromycin- and lincomycin-resistant colonies were selected on agar plates containing 1 μ g of erythromycin and 25 μ g of lincomycin per ml in the presence of 1 mM IPTG in order to allow production of *trxA* from

P_{SPAC}. The integration of pHVES1 into *trxA* was verified by PCR (data not shown), and the resulting strain was designated BIG1.

Radioactive labeling of cultures, 2-D protein gel electrophoresis, and N-terminal microsequencing. Cells grown in synthetic medium to an optical density at 500 nm of 0.4 were labeled for 3 min with 5 μ Ci of L-[³⁵S]methionine per ml before and 10 min after exposure to different stresses according to the method of Bernhardt et al. (5). For heat shock, the cells were shifted from 37 to 48°C. The other stress conditions were achieved by exposing the cells to either 4% (wt/vol) NaCl, 4% (vol/vol) ethanol, or 100 μ M paraquat. The different starvation conditions were provoked by cultivating the bacteria in a medium containing limiting amounts of glucose (0.05% [wt/vol]) or phosphate (0.3 mM). For labeling, samples were taken during the exponential or stationary growth phase. L-[³⁵S]methionine incorporation was stopped by the addition of chloramphenicol and an excess of cold methionine as well as by transferring the culture onto ice. The cells were disrupted by sonication, and crude protein extracts were prepared as described by Bernhardt et al. (5) Two-dimensional (2-D) polyacrylamide gel electrophoresis was performed with the Investigator system of Oxford Glycosystems (Oxford, England). Carrier ampholytes at a nonlinear gradient pH of 3 to 10 were used for the first dimension, and 12% acrylamide-bisacrylamide (30:0.8) was used for the second dimension. Each gel was loaded with a crude protein extract containing 2 \times 10⁶ cpm.

For microsequencing, the Coomassie blue-stained protein spots corresponding to thioredoxin were cut out from several 2-D gels and the collected gel pieces were concentrated, blotted onto a polyvinylidene difluoride membrane, stained, and sequenced as described previously (53).

General methods. Plasmid DNA isolation, cloning of DNA fragments, restriction enzyme analysis, and agarose gel electrophoresis were performed according to standard protocols (46). Transformation of competent *B. subtilis* cells was carried out by the method of Hoch (20). Determination of XylE activity before (37°C) and after (48°C) heat stress was described earlier (52).

Computer analysis of sequence data. The sequence data manipulations were performed with the Genetics Computer Group, Inc., sequence analysis software package.

Nucleotide sequence accession number. The nucleotide sequence of the regulatory region of *trxA* and the gene homologous to *xxa* reported in this paper appear in the EMBL and GenBank nucleotide sequence databases under the accession no. X79976 and X99275.

RESULTS

Nucleotide sequence of the regulatory region of *trxA*. In an attempt to analyze the heat shock response of *B. subtilis*, DNA fragments which confer heat induction of the promoterless

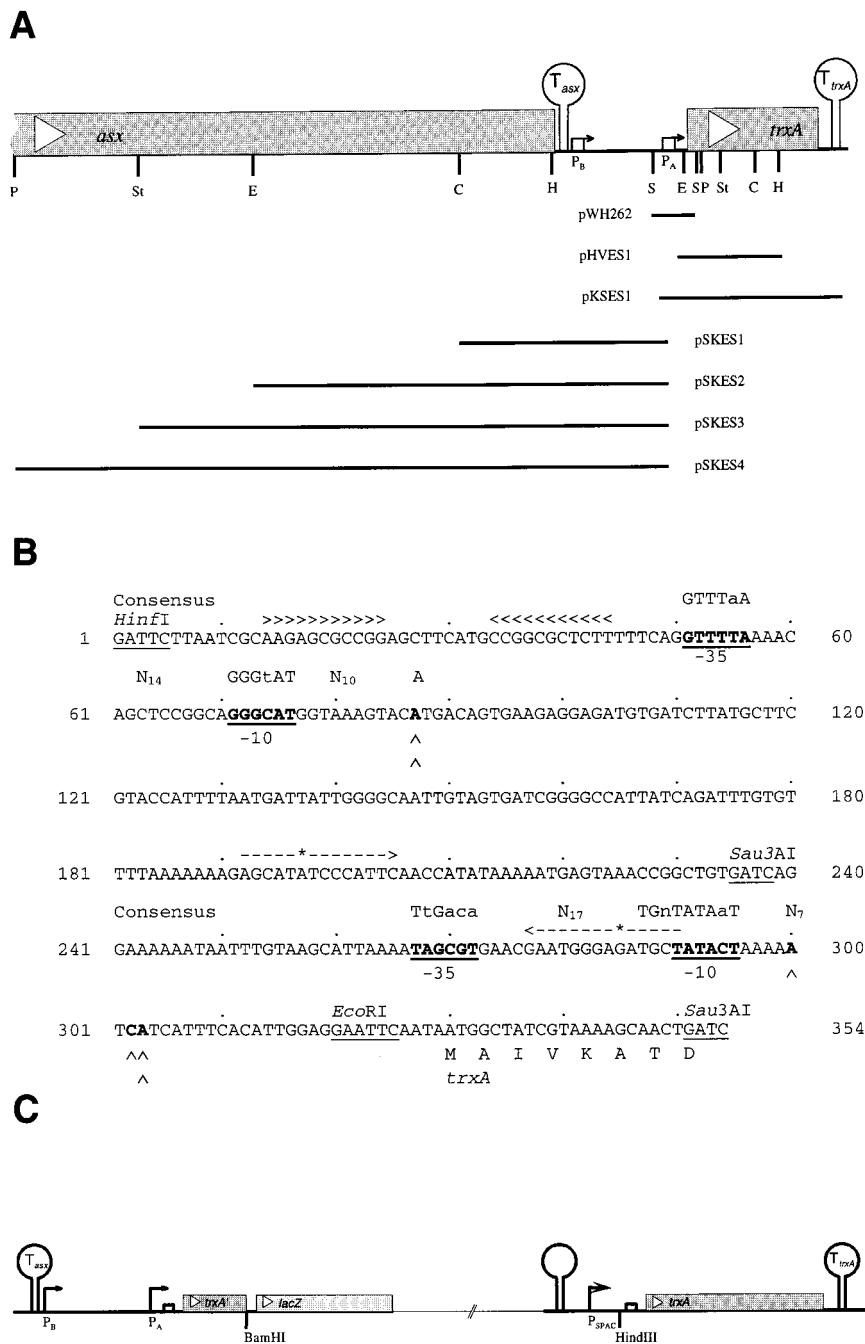


FIG. 1. Structure and organization of the *trxA* region of *B. subtilis*. (A) Schematic representation of the *trxA* region. The boxes indicate the locations of the coding region of *trxA* and an open reading frame with homology to the arabinosidase gene *xsA* from *B. ovatus* (57). Potential terminators (T) upstream and downstream of *trxA* and the promoters, P_B and P_A , which drive the expression of *trxA*, are labeled. The sites of the following restriction enzymes relevant for this study are marked: C, *Cla*I; E, *Eco*RI; H, *Hin*FI; P, *Pvu*II; S, *Sau*3AI; and St, *Sty*I. The lines represent the insertions of the plasmids used in this study. (B) Nucleotide sequence of the regulatory region of *trxA*. The potential -35 and -10 regions of both promoters are in boldface and underlined. The restriction sites used in this study are underlined. The potential 5' ends of the *trxA* transcripts are indicated by vertical arrowheads. The horizontal arrays of arrowheads label the potential terminator just upstream of the -35 region of the σ^B -dependent promoter, P_B . An inverted repeat overlapping with the potential promoter P_A is marked with arrows; the asterisks indicate a mismatch of the potential stem-loop structure. The consensus sequences for promoters recognized by RNA polymerase containing the vegetative sigma factor, σ^A (19), or the stress sigma factor, σ^B (18), are indicated, with positions critical for promoter recognition by the corresponding sigma factor in capital letters. (C) Schematic representation of the chromosomal rearrangement after integration of plasmid pHVES1 into the chromosome of *B. subtilis*.

reporter genes *xylE* and *cat* of the promoter probe vector pWH703 were cloned and characterized (52). One of the *Sau*3AI fragments contained at least part of the regulatory region, in addition to the beginning, of the coding region of

trxA encoding thioredoxin (8). Measurements of the activity of the reporter enzyme catechol-2,3-dioxygenase (encoded by *xylE*) and slot blot analysis of mRNA prepared before and after heat shock confirmed the heat induction of *xylE* and *cat*

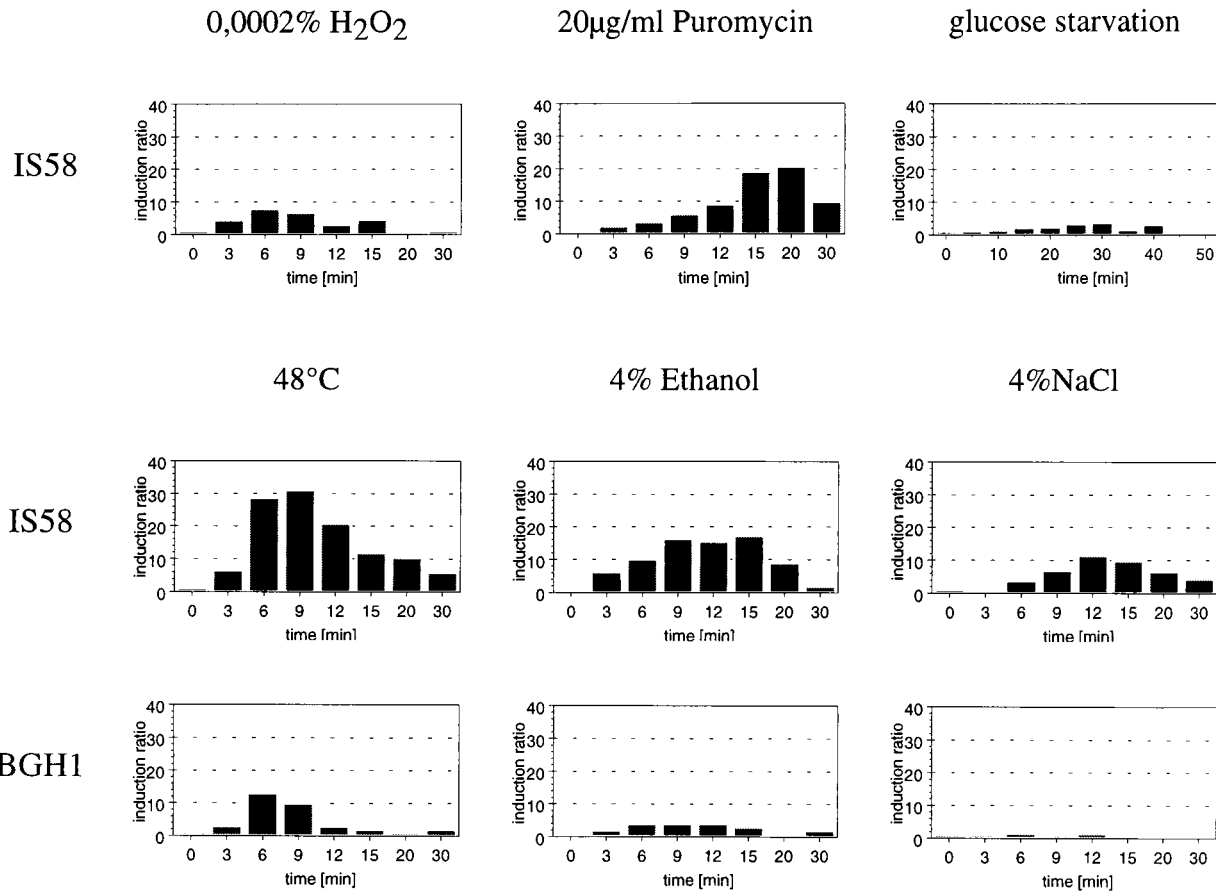


FIG. 2. Effect of various stresses on the *trxA* mRNA levels in *B. subtilis* IS58 (wild type) and the isogenic σ^B mutant (BGH1). Serial dilutions of total RNA prepared from *B. subtilis* before and at different times (3, 6, 9, 12, 15, 20, and 30 min) after exposure to stress were bound to a positively charged nylon membrane and hybridized with the digoxigenin-labeled antisense RNA probes specific for the *trxA* gene. The hybridization signals were quantified with a Personal Densitometer as described in Materials and Methods. The mRNA level in the control prior to stress was set to 1, and the induction ratios are shown.

conferred by the 120-bp *Sau3AI* fragment (data not shown). Inverse PCR was used to clone the whole regulatory region of *trxA*. The sequence of the 1.6-kb fragment revealed the presence upstream of *trxA* of a potential factor-independent terminator which is preceded by an open reading frame with significant similarity to the arabinosidase gene (*xsa*) from *Bacteroides ovatus* (57) (Fig. 1A and B). Since another, rho-independent potential terminator is located between *trxA* and *uvrB* (8), the sequence data suggested that *trxA* is transcribed as a single gene and does not form an operon with the flanking genes.

Transcriptional regulation of *trxA*. Since the initial experiments with the multicopy promoter probe vector already indicated heat induction at the *trxA* promoter, the effect of various stresses on the expression of *trxA* was measured by RNA slot blot analysis. In the wild-type strain, IS58, the level of *trxA* mRNA strongly increased after heat shock (30-fold), but it was also 8- to 20-fold higher after treatment with ethanol, salt, hydrogen peroxide, or puromycin (Fig. 2). Except with puromycin, the induction reached a maximum 6 to 12 min after the imposition of stress. The delay in response after treatment with puromycin might be attributed to the fact that puromycin fragments have to accumulate before they trigger the response. Starvation for glucose resulted in only a rather weak (three- to fourfold) induction (Fig. 2).

Induction by multiple stresses placed *trxA* in the group of general stress genes. Most of the general stress proteins of *B.*

subtilis require the stress sigma factor, σ^B , for their induction by various stresses (18). The induction of *trxA* in a strain with a deletion in *sigB* (BGH1) was reduced in response to heat and salt shock or ethanol stress (Fig. 2). Therefore, the heat, salt, and ethanol induction was at least partially controlled by σ^B . Induction by hydrogen peroxide and puromycin (Fig. 2) was not altered by the deletion of *sigB* (data not shown).

For promoter mapping, primer extension experiments were carried out with RNA from the wild-type strain (IS58) and a *sigB* mutant (BGH1). Two 5' ends of the *trxA* mRNA separated by 219 nucleotides were found (Fig. 1B and 3). The sequence elements preceding the upstream site, S_B , were very similar to known σ^B -dependent promoters (Fig. 1B), e.g., promoters of *sigB*, *ctc*, *gsiB*, *katE*, and *gspA* (18). This putative σ^B -dependent promoter, P_B , was not utilized in a *sigB* deletion strain (BGH1), confirming the suggestion that *trxA* induction was at least in part σ^B dependent (Fig. 3). The second, downstream 5' end of the *trxA* mRNA was not uniform and displayed, in addition to the major signal, two minor signals in the immediate vicinity. The sequence upstream of these three sites (S_A) resembled -10 boxes, which are recognized by RNA polymerase, containing the vegetative sigma factor, σ^A (19), but the -35 region did not resemble the -35 boxes of any of the known sigma factors of *B. subtilis* (17).

Both promoters appeared to be stress inducible. The σ^B -dependent promoter was strongly induced by heat and ethanol stress, but was induced to a lower extent by salt stress and

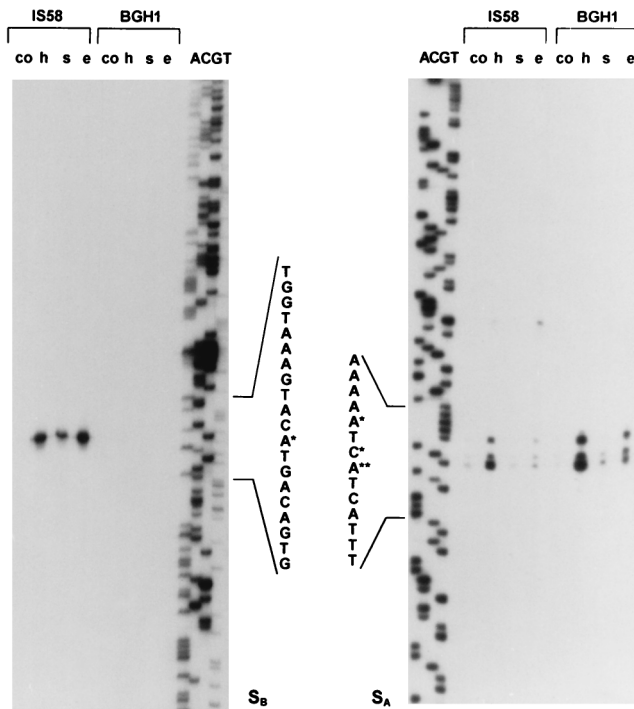


FIG. 3. Mapping of the 5' end of the *trxA* mRNA by primer extension analysis. Equal amounts of total RNA (5 μ g) isolated from *B. subtilis* IS58 (wild type) and the *sigB* mutant BGH1 before (co) and 9 min after exposure to heat shock (h), salt stress (s), and ethanol stress (e) served as templates. The 5' ends of the transcripts, S_B (left panel) and S_A (right panel), are marked with asterisks. Lanes A, C, G, and T show the dideoxy sequencing ladders obtained with the same primers used in the extension analysis (PtrxPE2 in the left panel and PtrxPE1 in the right panel).

glucose starvation (Fig. 3 and 4A and B). The putative σ^A -dependent promoter was strongly induced by heat stress and also by ethanol stress. In a *sigB* mutant only the intensity of the signals at S_A increased after stress (Fig. 3). The weak signal visible upstream of the signals at S_A in the wild-type strain after stress (Fig. 3) was located within the stem of the inverted repeat indicated in Fig. 1. This inverted repeat overlaps the putative promoter P_A and might be involved in the stress induction at S_A .

The Northern blot analysis proved that *trxA* is indeed transcribed as a monocistronic mRNA (Fig. 4) and allowed good resolution of both transcripts due to the different sizes of the mRNAs originating at S_B (594 bp) and S_A (377 bp). These data supported the results of the primer extension analysis. For exponentially growing cells, the 5'-end of the *trxA* transcript was mapped to S_A .

Although transcription initiating at both promoters was stress inducible, their induction profiles differed. The strongest *trxA* induction was mapped at the σ^B -dependent promoter after ethanol treatment and heat shock, whereas heat stress was more effective than ethanol treatment in inducing transcripts with the 5'-end S_A . Induction by salt stress was mostly confined to the σ^B -dependent promoter, explaining the almost complete absence of salt induction in a σ^B mutant (Fig. 2 and 3). In the absence of σ^B (BGH1), the induction by heat shock and ethanol stress at S_A was stronger than in the wild type, probably due to the lack of RNA polymerase competition for promoter binding (Fig. 4A).

Only the downstream promoter (with S_A at the 5' end), but not the σ^B -dependent promoter, was induced after treatment with puromycin or hydrogen peroxide (Fig. 4B). This result

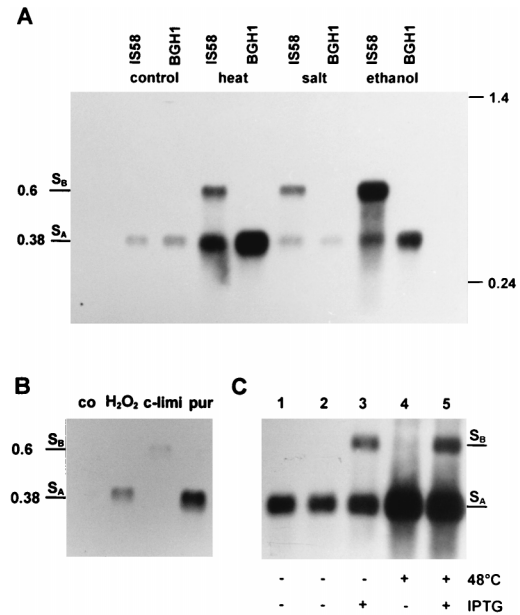


FIG. 4. Northern blot analysis. (A) RNA was isolated from *B. subtilis* IS58 (wild type) and BGH1 (*sigB*) at 37°C (control) and 9 min after a temperature shift to 48°C (heat) or after the addition of 4% NaCl (salt) or 4% ethanol (ethanol). The RNA was separated under denaturing conditions, transferred to a nylon membrane, and hybridized with a digoxigenin-labeled RNA probe specific for *trxA*. The locations of molecular size standards (in kilobases) and of mRNAs with 5' ends at S_B and S_A are marked. (B) Total RNA was isolated from the wild-type strain, IS58, during exponential growth (co), 9 min after exposure to 0.0002% H_2O_2 (H_2O_2) or 20 μ g of puromycin (pur)/ml, or 30 min after the cells entered the stationary phase as a result of the exhaustion of glucose (c-limi). The samples were processed as described for panel A. (C) RNA was isolated from *B. subtilis* BSA115 before (lane 1) or 6 min after exposure (+) to 48°C heat stress (lane 4), from bacteria grown in the absence (-) (lane 2) or presence (+) (lane 3) of IPTG, and after a combination of heat shock and the addition of IPTG (lane 5).

explains why the same induction ratios of *trxA* mRNA were found in the wild type and in the σ^B mutant after treatment with puromycin or H_2O_2 in slot blot hybridizations (data not shown).

If the activation of σ^B was solely responsible for the induction of *trxA* at S_B , production of active σ^B in the absence of stress should have resulted in increased transcription from the σ^B -dependent promoter. In the *B. subtilis* strain BSA115 (54), in which expression of *sigB* is controlled by P_{SPAC} and the anti-sigma factor RsbW (4) is not produced because of a frameshift mutation in *rsbW*, the addition of IPTG triggers the production of active σ^B molecules. In this strain only the upstream promoter, P_B , was induced in response to IPTG addition (Fig. 4C). Heat shock without the addition of IPTG induced the downstream start site, S_A , only, because this strain does not produce active σ^B in response to stress (55). Both promoters were induced in heat-shocked cells of BSA115 treated with IPTG (Fig. 4C).

In order to exclude the possibility that the induction of *trxA* in response to stress was due to a stabilization of the mRNA during stress, exponentially growing cells were treated with rifampin and the influence of heat shock on the amount of *trxA* mRNA was analyzed. In Northern blot as well as in slot blot experiments the heat shock did not significantly change the half-life of the *trxA* mRNA (data not shown). The increase in the level of the *trxA* mRNA must therefore be due to enhanced transcriptional initiation.

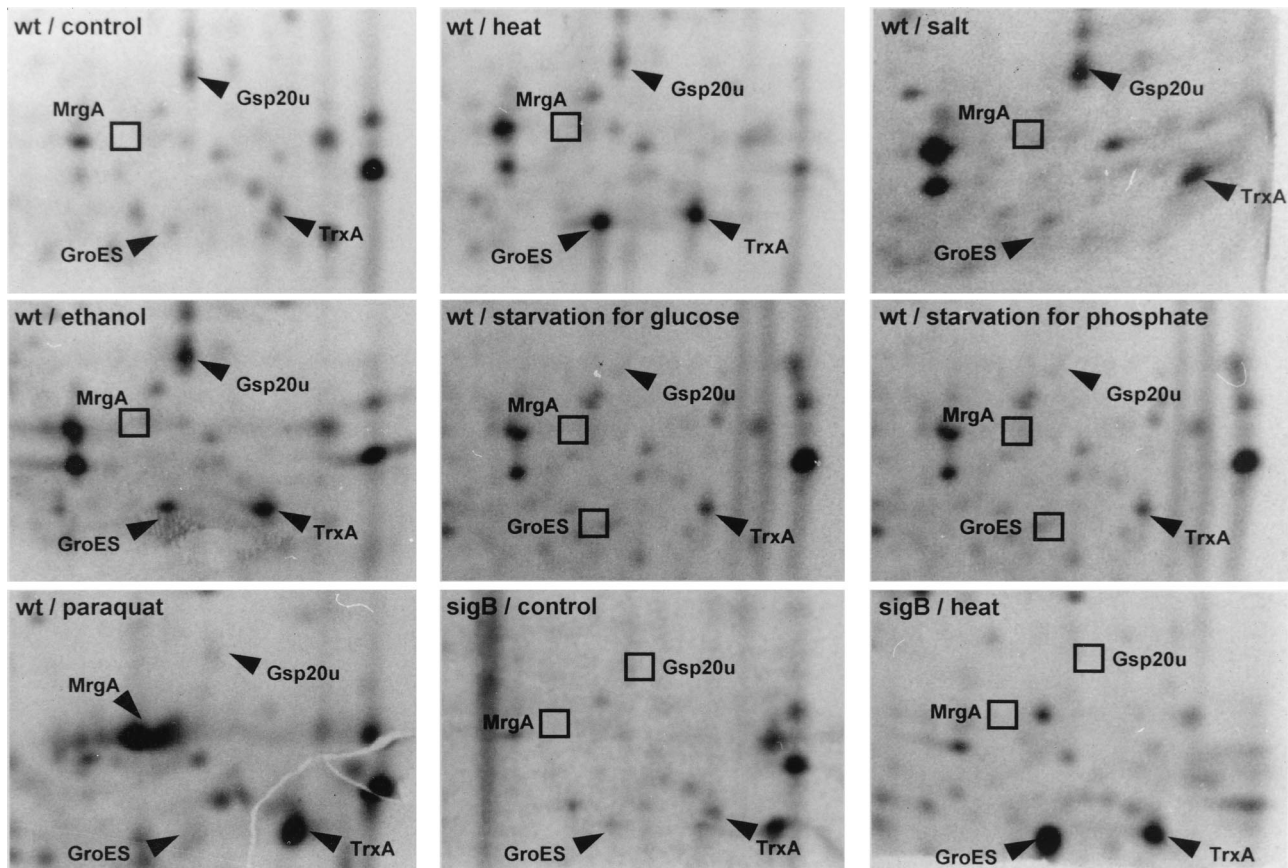


FIG. 5. Synthesis of thioredoxin during exponential growth and after the imposition of different stresses and starvation. Wild-type bacteria (wt) and a strain with a null mutation in *sigB* (*sigB*) were grown in a synthetic medium, exposed to the indicated stimuli, and labeled prior to (control) or after stress as described in Materials and Methods. Crude protein extracts were prepared and separated by 2-D protein gel electrophoresis. The sections of the autoradiograms containing thioredoxin (TrxA) are displayed. The general stress protein Gsp20U, the oxidative stress-specific protein MrgA, and the rather heat-specific protein GroES are indicated as marker spots.

Identification of TrxA on 2-D protein gels and synthesis of thioredoxin after stress and starvation. The determination of the N-terminal sequences of stress proteins enabled the identification of TrxA on 2-D protein gels. The N-terminal sequence MAIVKATDQSFSAETSEGVVLA perfectly matched the predicted amino acid sequence of TrxA and proved that the ATG codon marked by Chen et al. (8) is indeed the start codon of *trxA*. After the spot corresponding to thioredoxin on 2-D protein gels was identified, the influence of stress and starvation on the synthesis of thioredoxin was analyzed by 2-D gel electrophoresis (Fig. 5). Although synthesis of thioredoxin was already detected during growth, exposing cells to heat, salt, ethanol, or paraquat, a very effective inducer of oxidative stress proteins in *B. subtilis*, clearly induced the synthesis of thioredoxin. The induction of thioredoxin synthesis by glucose or phosphate starvation was less pronounced. Deleting the gene encoding the stress sigma factor, σ^B , did not abolish the heat induction of thioredoxin, reinforcing the hypothesis that the second promoter is able to compensate for the loss of σ^B in heat induction.

Construction of *trxA* mutants. We repeatedly failed to construct an insertional mutant of *trxA* and decided to construct a conditional mutant by placing *trxA* under the control of P_{SPAC} as described in Materials and Methods (Fig. 1C). In this strain (BIG1) the level of thioredoxin is controlled by the amount of IPTG added. The strain requires IPTG for growth, which

stopped upon removal of IPTG (Fig. 6). These data argued that thioredoxin is an essential protein of *B. subtilis*.

DISCUSSION

Thioredoxin seems to be an essential protein of *B. subtilis*, in contrast to *E. coli* (21, 34), since we failed to disrupt the *trxA* gene, and removal of IPTG from a strain expressing thioredoxin from an IPTG-controlled promoter resulted in growth arrest. Recently, thioredoxin has also been shown to be essential in *Synechocystis* (38) and *Rhodobacter sphaeroides* (40), and growth becomes dependent on the presence of thioredoxin in *S. cerevisiae* lacking glutathione reductase (37). Therefore, in *B. subtilis* thioredoxin might serve multiple functions in vivo, some of which in *E. coli* can also be catalyzed by glutathione and glutaredoxin (22), a system which has not yet been characterized in *B. subtilis*.

In this report we identify thioredoxin as a heat shock protein in *B. subtilis*. Previously, thioredoxin had been observed to induce heat shock only in human cells (24). In *B. subtilis*, three classes of heat-inducible proteins have been described (18). Class I genes, as exemplified by the *dnaK* and *groE* operons, are mainly induced by heat stress. Their heat induction involves a σ^A -dependent promoter, an inverted repeat (CIRCE, for controlling inverted repeat of chaperone expression [TTAGCACTC-N₉-GAGTGCTAA]) that is highly conserved

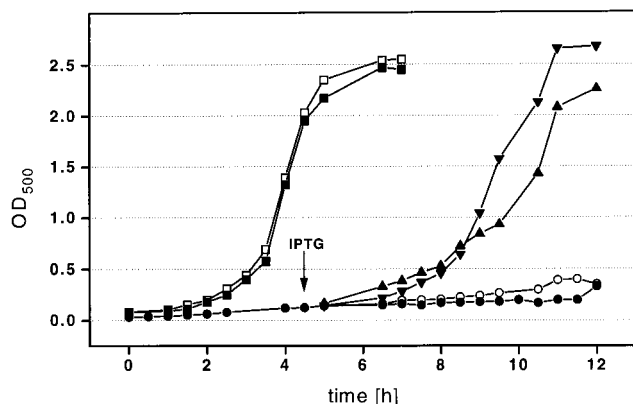


FIG. 6. Effect of variations in the level of TrxA on the growth of *B. subtilis*. The *B. subtilis* strain BIG1, in which the expression of *trxA* is controlled by P_{SPAC} was grown in the presence of 50 μ M IPTG and diluted 30-fold into fresh prewarmed Luria broth with or without 50 μ M IPTG. At the time indicated by the arrow different concentrations of IPTG were added to the cultures incubated without IPTG. The wild-type strain, IS58, is included as a control. ■, IS58 control; □, BIG1 with 50 μ M IPTG added immediately; ●, BIG1 without IPTG; ○, BIG1 with 5 μ M IPTG; ▲, BIG1 with 10 μ M IPTG; ▼, BIG1 with 50 μ M IPTG. OD₅₀₀, optical density at 500 nm.

among eubacteria, and a repressor interacting with the CIRCE element (59, 60). The activity of this repressor is modified by the GroE chaperonin machine (35).

Most of the heat-inducible genes of *B. subtilis* are also induced by a diverse range of stress conditions, such as salt stress, ethanol stress, and starvation for glucose, phosphate, or oxygen. These stress- or starvation-inducible proteins are called general or nonspecific stress proteins (43), the majority of which absolutely require σ^B for their induction by various stresses (class II [18, 53]). Only a few genes, including *lon*, *clpC*, *clpP*, *clpX*, and *ftsH*, remain inducible by different stress conditions in the absence of σ^B (class III [see reference 18 for a review]).

trxA remained stress inducible in the σ^B mutant, although at a reduced level, and therefore belongs to the class III heat shock genes. Transcription of *trxA* initiates at two different promoters: the upstream promoter is σ^B dependent, while the downstream promoter is presumably σ^A dependent. The potentially σ^A -dependent promoter might require activation by a positive regulatory protein, since it deviated in four of six positions from the consensus sequence of the -35 region recognized by σ^A . Both promoters were heat and stress inducible, explaining the observation that *trxA* remained stress inducible even in a *sigB* mutant. This heat and stress induction occurring at a putatively σ^A -dependent promoter in the absence of a CIRCE element is typical of the class III heat shock genes. It is interesting to note that the stress induction patterns at the two start sites differed. Both are induced by heat and ethanol stresses, although to a different extent, but only the transcription starting at the downstream start site, S_A , is induced in cells treated with puromycin, paraquat, or, to a lower extent, hydrogen peroxide.

A similar double control by heat and stress involving a σ^B -dependent promoter as well as a second, putatively σ^A -dependent promoter was also described for *clpC*, a presumable chaperone and subunit of a stress protease (25). The stress induction patterns of the downstream, potentially σ^A -dependent promoters of the *trxA* and *clpC* genes are quite similar. Therefore, it is tempting to speculate that ClpC and thioredoxin of *B. subtilis*, both of which might participate in the

folding and refolding of proteins, are induced by heat and other stresses through similar mechanisms. Recently we identified a regulatory protein which might participate in the induction of the *clpC* operon of *B. subtilis* (26). The possible function of this regulator in the expression of *trxA* remains to be elucidated.

The thioredoxin-encoding gene *trxA* of *B. subtilis* is induced by H_2O_2 and paraquat at the downstream site, S_A , indicating that thioredoxin is involved in the maintenance of the protein structure under oxidative stress, as suggested for thioredoxin and thioredoxin reductase in *Mycobacteria* (58), yeast (27, 36), and endothelial cells (14). Because any exposure of cellular proteins to oxidative stress may lead to an inappropriate formation of disulfide bonds, the protection of proteins in a functional state may require reduction of disulfides even in the otherwise very reductive cytoplasm of bacteria (9). Enhanced disulfide bond formation occurs in the cytoplasm of *E. coli* with a mutation in thioredoxin reductase (9). Furthermore, another repair mechanism of proteins damaged by oxidative stress is known for *E. coli*: the enzyme methionine sulfoxide reductase is able to recognize methionine sulfoxide and to reduce it to methionine. This repair reaction requires thioredoxin as a substrate (42).

In addition to oxidative stress, *trxA* is also induced by other stresses, including heat shock (Fig. 2 to 5). Heat shock and ethanol might both enhance incomplete reduction of molecular oxygen by respiration and therefore generate higher levels of peroxide anions. Although induction of *trxA* by heat stress or ethanol at the σ^A -dependent promoter could be the result of the increased formation of reactive oxygen species, the signal responsible for induction at the σ^B -dependent promoter must be different because oxidative stress does not induce the *sigB* regulon. Analysis of the thioredoxin function following stress might help to reveal the role of the σ^B -dependent general stress response of a nongrowing cell. Although more than 40 σ^B -dependent general stress proteins have been described (5, 53), there is only limited information available on the function of these proteins under stress and starvation. Recently, we obtained evidence that σ^B is required for the nonspecific development of resistance to hydrogen peroxide in nongrowing, glucose-starved cells without any prior exposure to oxidative stress (13) and for the development of resistance against cumene hydroperoxide (1).

The double control at σ^A -dependent and σ^B -dependent promoters of genes like *trxA* and *clpC* ensures a specific induction by oxidative stress during growth as well as a nonspecific and protective induction in the nongrowing or stressed cells by σ^B . The σ^B -dependent general stress response of *B. subtilis* comprises other genes in addition to *clpC* (23) and *trxA*, such as the catalase-encoding *katE* (12), *clpP* (15), and *dps* (2), which are suspected to be related to oxidative stress and to protect the cell at different levels against oxidative damage. First, enzymes like catalase II (KatE) are produced and help to destroy reactive oxygen species. Secondly, ClpC and TrxA are induced at least partially in a σ^B -dependent mechanism and raise the capacity of the bacteria to recover proteins damaged by oxidative stress. The risk of potential damage to the DNA is reduced by the induction of the nonspecific DNA-binding and -protecting protein Dps, which has recently been shown to play a crucial role in the development of nonspecific starvation-mediated resistance to oxidative stress (2). Finally, proteases like ClpP (15) and the chaperone and ATPase ClpC might be produced to degrade irreversibly damaged proteins and to recycle the chaperones bound to these proteins.

A similar function in the control of the expression of sta-

tionary-phase and stress genes has been assigned to RpoS in *E. coli*. The DNA-protecting protein Dps and a catalase (KatE), as well as a glutathione oxidoreductase (Gor), which have been shown to be subject to RpoS-dependent regulation, are essential to the oxidative stress resistance acquired in the stationary phase of growth (3, 11). In yeast cells, also, there is an increased requirement for protection from oxidative stress as the cells enter stationary phase (16). In *Schizosaccharomyces pombe*, glutathione reductase is not only induced by redox-cycling agents but also by high osmolarity, heat shock, or stationary phase, indicating that all these factors might provoke oxidative stress (28).

These observations raise the possibility that one of the important functions of the nonspecific stationary phase and stress response (of *B. subtilis*, *E. coli*, and probably also *S. cerevisiae*) may be the protection of the nongrowing or stressed cells from damage by reactive oxygen species.

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