JOURNAL OF BACTERIOLOGY, Nov. 1996, p. 6571–6578 Vol. 178, No. 22
0021-9193/96/$04.00+0

General and Oxidative Stress Responses in *Bacillus subtilis*: Cloning, Expression, and Mutation of the Alkyl Hydroperoxide Reductase Operon

HAIKE ANTELMANN,[1] SUSANNE ENGELMANN,[1] ROLAND SCHMID,[2] AND MICHAEL HECKER[1]*

Institut für Mikrobiologie und Molekularbiologie, Ernst-Moritz-Arndt-Universität, 17487 Greifswald, Germany,[1] *and Abteilung für Mikrobiologie, Universität Osnabrück, 49076 Osnabrück, Germany*[2]

Received 7 May 1996/Accepted 4 September 1996

The AhpC subunit of the *Bacillus subtilis* alkyl hydroperoxide reductase was identified as a general stress protein induced in response to heat or salt stress or after entry of the organism into the stationary phase. The *ahp* operon, encoding the two subunits AhpC and AhpF, was cloned and localized between the *gntRKPZ* operon and the *bglA* locus. Two-dimensional gel analyses revealed an especially strong induction of AhpC and AhpF in cells subjected to oxidative stress. Transcriptional studies showed a 3- to 4-fold induction of *ahp* mRNA after heat or salt stress or starvation for glucose and a 20-fold induction by oxidative stress, thus confirming the protein induction data for AhpC and AhpF. Stress induction occurred at a σ^A-dependent promoter that overlaps with operator sites similar to the per box. Compared with the wild type, the *ahpC* mutant was resistant to hydrogen peroxide because of the derepression of the peroxide regulon (N. Bsat, L. Chen, and J. D. Helmann, J. Bacteriol. 178:6579–6586, 1996) but more sensitive to cumene hydroperoxide (CHP) during exponential growth. In contrast, stationary-phase wild-type and *ahpC* mutant cells displayed complete resistance to treatment with 1 mM CHP. Moreover, a σ^B mutant was found to be extremely sensitive to CHP during vegetative growth and in stationary phase, which indicates that σ^B-dependent general stress proteins are involved in the protection of cells against oxidative stress.

In their natural environment, *Bacillus subtilis* cells are frequently faced with different growth-restricting conditions, like heat, salt, and oxidative stress and deprivation of nutrients. To overcome these adverse conditions, *B. subtilis* cells have developed a network of adaptive responses. One response to different stress conditions is the induction of a distinct group of proteins that are visible on two-dimensional (2-D) protein gels, referred to as general stress proteins (GSPs) (30, 31, 57). It is evident that the majority of the general stress genes belong to the large σ^B-dependent stress or stationary-phase regulon, since the proteins are not stress inducible in a σ^B deletion mutant (10, 30, 57). Because of their coordinate induction pattern, they are thought to provide the cell with nonspecific protection under adverse conditions (30). There are only a few GSPs, like ClpC, ClpP, ClpX, TrxA, Lon, and FtsH, which can be induced by stress independently of σ^B (18, 26, 27, 30, 37, 47, 50). Besides these general stress genes, each single stressor induces its specific set of stress-specific proteins. The chaperone operons *dnaK* (59) and *groESL* (38, 51, 57) are induced specifically after heat stress. Their thermoregulation requires the major vegetative sigma factor σ^A, a conserved inverted repeat (CIRCE) upstream of the translational start points, and a repressor (ORF39) that interacts with the CIRCE element (61, 62, 64).

Pretreatment of exponentially growing *B. subtilis* cells with sublethal (protective) doses of H_2O_2 confers resistance to a lethal H_2O_2 treatment (20, 44). As shown by one-dimensional gel electrophoresis, this inducible response is related to the synthesis of a specific group of proteins (19, 28, 44), which differs significantly from the GSPs (6, 30). Only a few of these hydrogen peroxide-inducible proteins, including the two subunits of the alkyl hydroperoxide reductase (AhpC and AhpF) and a DNA-binding and protecting protein (MrgA), seem to be slightly induced by heat shock (15, 16, 19, 44). Together with the vegetative catalase, KatA, and heme biosynthesis proteins, they constitute a peroxide regulon whose induction provides exponentially growing pretreated cells with specific protection against an otherwise lethal H_2O_2 challenge (17, 19).

In this study, we identified one of the σ^B-independent GSPs as the AhpC subunit of the *B. subtilis* alkyl hydroperoxide reductase. We describe the cloning of the *B. subtilis ahp* operon and the regulation of the expression of this operon in response to general and oxidative stress conditions. In addition to the specific function of Ahp in the detoxification of organic hydroperoxides, σ^B-dependent GSPs seem to be involved in the protection of growing and starving cells against cumene hydroperoxide (CHP).

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH5α and RR1 were routinely grown in a complex medium and used as hosts for DNA manipulation. *B. subtilis* strains were cultivated under vigorous agitation at 37°C in a synthetic medium described previously (54). Exponentially growing cells were exposed to different stress conditions in accordance with the procedure of Völker et al. (57). For heat shocking, cells were shifted from 37 to 50°C. The other stress conditions were achieved by exposing cells to either 4% (wt/vol) NaCl, 60 μM H_2O_2, or 100 μM CHP. Starvation for glucose was provoked by cultivating the bacteria in a medium containing limiting amounts of glucose (0.05%, wt/vol).

Survival assays after H_2O_2 and CHP challenge. Cells grown in a minimal medium to an optical density at 500 nm of 0.4 were transferred to 100-ml Erlenmeyer flasks, and H_2O_2 was added to various concentrations from 100 μM to 20 mM. Samples subjected to two H_2O_2 treatments were treated sequentially for 15 min each. For CHP challenge, exponentially growing cells (10 ml each) were placed in 100-ml Erlenmeyer flasks containing various concentrations of CHP in the range from 100 μM to 1 mM (diluted in dimethyl sulfoxide). For determination of the CHP sensitivity along the growth curve, cultures were grown under glucose starvation conditions and placed in Erlenmeyer flasks containing 1 mM CHP at various time points (see Fig. 5). All tubes were shaken at 200 rpm for 15 min at 37°C. Survival of the cells was determined by plating

* Corresponding author. Phone: 03834/864200. Fax: 03834/864202. Electronic mail address: hecker@microbio7.biologie.uni-greifswald.de.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
Strains		
E. coli		
DH5α	F$^-$ ϕ80d *lacZ* ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 gyrA96*	48
RR1	F$^-$ *mcrB mrr hsdS20* (r_B^- m_B^-) *ara14 proA2 lacY1 leu galK2 rpsL20* (Sm^r) *xyl5 mtl1 supE44*	48
B. subtilis		
IS58	*trpC2 lys-3*	52
168	*trpC2*	3
BGH1	*trpC2 lys-3 sigB*::Δ*Hin*dIII-*Eco*RV::*cat*	42
ML6	*trpC2 sigB*::Δ*Hin*dIII-*Eco*RV::*cat*	33
BAhpC	*trpC2 lys-3 ahpC*::pJAHP9	This work
HB1022	*trpC2 mrgA*::Tn*917-lacZ* (MLS^r) attSPβ	16
Plasmids		
pBluescript II KS(−)	Cloning vector; Ap^r	Stratagene
pJH101	Integrative plasmid; Ap^r Tc^r Cm^r	24
pAHP9	pBluescript II KS(−) containing the 493-bp PCR product of *ahpC*	This work
pJAHP9	pJH101 containing the 520-bp *Bam*HI-*Sal*I fragment of pAHP9	This work
pJAHP11	Obtained after plasmid rescue from *Nde*I-digested DNA of *B. subtilis* BAhpC	This work
pJAHP15	Obtained after plasmid rescue from *Aat*II-digested DNA of *B. subtilis* BAhpC	This work
pJAHP12	pBluescript II KS(−) containing the 762-bp *Eco*RI-*Eco*RI fragment of pJAHP11	This work
pJAHP13	pBluescript II KS(−) containing the 876-bp *Eco*RI-*Eco*RI fragment of pJAHP11	This work

appropriate dilutions on Luria-Bertani agar at 37°C and comparing the viable counts with those of a control tube shaken for the same period of time.

Analytical and preparative 2-D PAGE and N-terminal sequencing. Analytical 2-D polyacrylamide gel electrophoresis (2-D PAGE) was performed with bacterial cell extracts which were labelled with L-[^{35}S]methionine for 3 min before and 5 min after exposure to oxidative stress in accordance with the procedure of Hecker et al. (29). For N-terminal sequencing, protein samples were separated by preparative 2-D PAGE with the 2-D electrophoresis system of the Millipore Corporation, and the protein spots collected from several 2-D gels were concentrated as described previously (4). The protein was blotted onto a polyvinylidene difluoride membrane, stained, and sequenced as described previously (4). A modification of the standard cycle was used for faster sequencing.

Cloning and sequencing of *ahp* and construction of an *ahp* insertional mutant. Taking into account the codon usage of *B. subtilis* (58), two degenerate oligonucleotide PCR primers, 5′ AAAGAAGTNYTNCCDTWYGAAGC 3′ (P1) and 5′ ATTTNGCHGGRCANACTTCNCC 3′ (P2), were deduced from the AhpC N-terminal sequence, KEVLPFEA, and from a C-terminal conserved catalytic motif, GEVCPAKW, respectively. A 493-bp PCR product was amplified from chromosomal DNA of *B. subtilis* IS58 and cloned into the *Eco*RV site of the plasmid pBluescript II KS(−), resulting in plasmids with the cloned sequence in opposite directions (pAHP7 and pAHP9). Plasmids were sequenced by the dideoxy-chain termination method of Sanger et al. (49). The 520-bp *Bam*HI-*Sal*I fragment of pAHP9 was ligated with the insertional vector pJH101 which had been digested with the same enzymes. The resulting plasmid, pJAHP9, was used to transform competent cells of *B. subtilis* IS58. Chloramphenicol-resistant colonies were selected on agar plates containing 5 μg of chloramphenicol per ml. The integration was verified by Southern blot analysis. To clone the promoter region of *ahpC* and the downstream *ahpF* gene, chromosomal DNA of the resulting *ahpC* mutant strain BAhpC was digested with *Aat*II and *Nde*I, religated, and introduced into *E. coli* DH5α, creating plasmids pJAHP15 and pJAHP11. For sequencing, a *Dra*I-*Sal*I fragment of pJAHP15 and two *Eco*RI fragments (762 and 876 bp) of pJAHP11 were subcloned. The sequence was determined from a set of subclones and by primer walking.

Analysis of transcription. Total RNA of each of the *B. subtilis* strains under investigation was isolated from exponentially growing cells before and after exposure to stress by the acid phenol method of Majumdar et al. (40) with modifications as described previously (57). Serial dilutions of total RNA were transferred onto a positively charged nylon membrane by slot blotting and hybridized with digoxigenin-labeled RNA probes in accordance with the instructions of the manufacturer (Boehringer Mannheim). The chemiluminograms were quantified with a personal densitometer from Molecular Dynamics. Induction values were calculated by setting the value of the control to 1.

Northern (RNA) blot analyses were performed as described previously (59). Hybridization specific for *ahpC* was conducted with a digoxigenin-labeled RNA probe synthesized in vitro with T7 RNA polymerase (noncoding strand) from *Hin*dIII-linearized plasmid pAHP7 containing the 493-bp fragment of *ahpC*.

For primer extension experiments, the synthetic oligonucleotide primer complementary to the N-terminus-encoding region of the *ahpC* gene (PAHP1, 5′ AATGGAAGTACTTCTTTACCG 3′) was 5′-end labelled with [γ-^{32}P]ATP and used as the primer for the primer extension analysis as described previously (59).

Electron microscopic analysis. *B. subtilis* IS58 and BAhpC were grown in minimal medium as described above. The flagella of cells from exponential- and stationary-phase cultures were visualized by negative staining with 1% uranyl acetate, using glow-discharged 400-mesh grids with carbon-coated pioloform films. Samples were examined at 80 kV with a Zeiss EM 906 electron microscope.

General methods. Plasmid isolation, restriction enzyme analysis, transformation of *E. coli*, ligation of DNA fragments, and filling in of the recessed 3′ termini by the Klenow fragment of DNA polymerase I were performed in accordance with the standard protocols (48). Chromosomal DNA from *B. subtilis* was isolated as described by Meade et al. (43). Transformation of natural competent *B. subtilis* cells was carried out as described by Hoch (32).

Computer analysis of sequence data. The sequence data manipulations were performed with the Genetics Computer Group sequence analysis software package. Database searches were performed with the Blast program (1).

RESULTS

Identification of AhpC by N-terminal sequencing. Among the numerous GSPs identified by 2-D gel electrophoresis and subjected to N-terminal sequencing, we found a polypeptide (Gsp22) whose sequence over the first 28 amino acids was 57% identical to the small subunit of the alkyl hydroperoxide reductase from *E. coli* and *Salmonella typhimurium* (AhpC) (56) and was completely identical to the N terminus of the previously identified AhpC protein of *B. subtilis* (28). Because this protein was previously shown to be induced by oxidative stress (28) as well as by heat and salt stress (57), we studied the regulation and function of the corresponding gene in response to stress.

Cloning and sequence analysis of the *ahp* operon. AhpC homologs are widely distributed among organisms of all kingdoms and define a large family of more than 25 proteins, the thiol-specific antioxidant protein family (12, 13). The most common features of this group are related to one or two conserved cysteine-containing hydrophobic motifs (VCP) responsible for the catalytic antioxidative activity (14). For cloning of the *B. subtilis ahpC* gene, we derived degenerate oligonucleotide primers corresponding to the N-terminal sequence of AhpC and the C-terminal conserved cysteine-containing catalytic motif and amplified a 493-bp PCR product by using these primers with chromosomal *B. subtilis* IS58 DNA as the template. This *ahpC* fragment was cloned into an integrational vector which was integrated into the chromosomal *ahpC* gene,

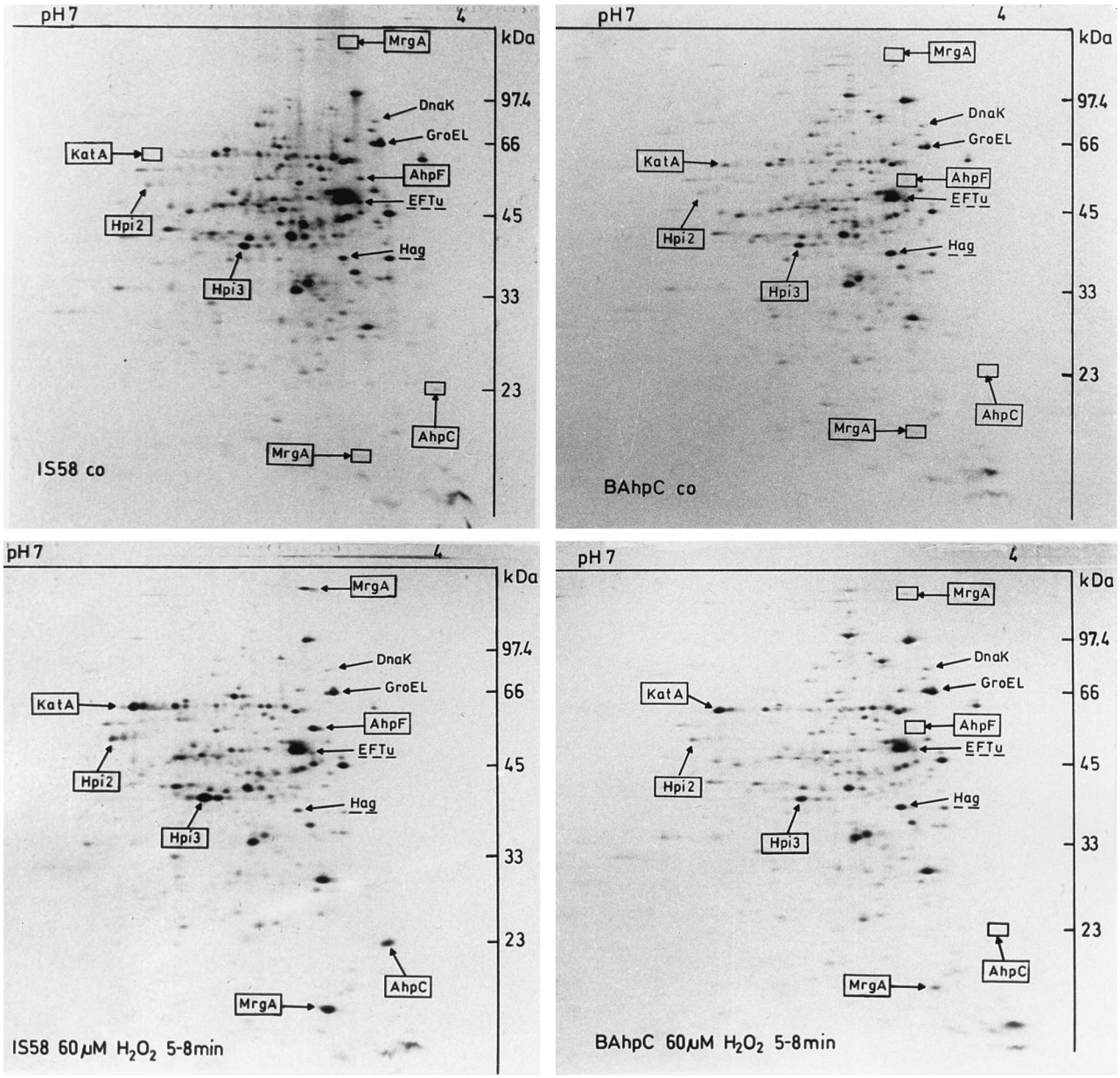

FIG. 1. Synthesis of proteins in *B. subtilis* wild-type (IS58) and *ahpC* mutant (BAhpC) strains after treatment with 60 μM H_2O_2. Bacteria were pulse-labelled for 3 min with L-[^{35}S]methionine before (co) and 5 min after H_2O_2 challenge (60 μM H_2O_2, 5 to 8 min). Proteins induced by H_2O_2 (Hpi2 and -3, AhpC, AhpF, MrgA, and KatA) are boxed.

resulting in an *ahpC* disruption mutant, BAhpC. The *ahp* operon, including the flanking noncoding regions, was cloned by plasmid rescue of chromosomal BAhpC DNA. The nucleotide sequences of *ahpC* and the 5′ end of *ahpF* were determined in the 930-bp *Eco*RI-*Hin*dIII interval from plasmids pJAHP12 and pJAHP13. The sequence downstream of the *Hin*dIII site was identical to the sequence of the C-terminal part of the NADH dehydrogenase (*ndh*) published by Zhang and Aronson (63). The nucleotide sequence of the presumed promoter region was determined from the *Dra*I-*Eco*RI fragment of plasmid pJAHP15, which contained the C-terminal region of the *gntRKPZ* operon as reported by Fujita et al. (25). This result was in agreement with the map position for *ndh* or *ahpF* at 340° on the *B. subtilis* chromosome (2, 63).

While this work was in progress, we learned that the *B. subtilis ahp* operon has been independently identified and cloned by Bsat et al. (11) and sequenced by Kasahara et al. (GenBank accession number D78193) (35) as part of the *B. subtilis* genome sequencing project.

Nucleotide sequence analysis revealed two open reading frames (ORF1 and ORF2) of 563 and 1,429 bp whose predicted products were highly similar to the *E. coli* and *S. typhimurium* AhpC and AhpF proteins (56). Furthermore, the translation products of the first codons of ORF1 (except for Met) and ORF2 were identical to the N-terminal amino acid sequences of AhpC and AhpF as determined by Hartford and Dowds (28), and therefore we designated ORF1 as *ahpC* and ORF2 as *ahpF*. The *B. subtilis ahpC* gene has the capacity to

code for a protein of 187 amino acids with a predicted molecular mass of 20,482 Da and a calculated pI of 4.28. The gene is preceded by a potential ribosome binding site (5′ GGAGGA 3′) located 8 nucleotides upstream of the start methionine codon (ATG). The *ahpF* gene is located downstream of *ahpC* and is predicted to encode a 509-amino-acid protein with a molecular mass of 54,705 Da and an isoelectric point of 4.7. The translation initiation codon TTG of the *ahpF* gene is preceded by a Shine-Dalgarno sequence 8 nucleotides upstream (5′ GGAGTG 3′). Two potential transcription termination signals were previously identified downstream of *ahpF* (63).

The deduced amino acid sequences of *ahpC* and *ahpF* exhibited strong identity to the NADH dehydrogenase operon of *Bacillus alcalophilus* (83%) (60), the NADH oxidase operon of *Amphibacillus xylanus* (87 and 72%) (45), AhpC of *Staphylococcus aureus* (78%) (5), the Ahp operon of *S. typhimurium* (66 and 56%) (56), ORF C and ORF A of the *Clostridium pasteurianum* rubredoxin operon (31%) (41), and AhpC homologs of *Corynebacterium diphtheriae* (26%) (55) and other gram-positive microorganisms. Furthermore, it has been shown that AhpF and thioredoxin reductase define a distinct family of disulfide-containing oxidoreductase proteins (13, 56). The highly conserved redox-active cysteine region of AhpF was found at amino acid positions 337 to 342. Further comparison revealed the presence of an N-terminal hydrophobic membrane binding motif, two conserved adenine binding sites for flavin adenine dinucleotide (210 to 241) and NADH (349 to 379), and a C-terminal flavin binding site for flavin adenine dinucleotide (469 to 479).

2-D gel analysis of oxidatively stressed wild-type and *ahpC* mutant cells. The 2-D gel pattern of proteins synthesized after exposure of *B. subtilis* wild-type cells to sublethal doses of H_2O_2 differs significantly from that seen in response to general stress conditions, like heat, salt, or ethanol stress (6, 30). As seen in Fig. 1, the synthesis of at least seven proteins, of 16, 23, 41, 50, 54, 59.5, and 110 kDa, was increased by H_2O_2 in the wild-type strain. Among these proteins, the 23-kDa AhpC was strongly induced by oxidative stress in the wild-type strain and eliminated from 2-D gels of the *ahpC* mutant. Furthermore, the 54-kDa protein was identified as the AhpF protein on the 2-D gels. This protein was induced coordinately with AhpC in the wild type and was not detectable in an *ahpC* mutant, presumably because the insertion mutation is polar. Both proteins, AhpC and AhpF, were found to be induced by heat and salt stress (6) and, therefore, display an overlapping response to H_2O_2 and general stress conditions.

Besides AhpC and AhpF, the 59.5-kDa protein, which was identified as the vegetative catalase KatA, was strongly induced by H_2O_2 in the wild type (22). KatA was still induced in the *ahpC* mutant, and, compared with the wild type, the basal level of KatA was slightly increased, confirming the results presented in the accompanying paper (11).

In addition, two H_2O_2-inducible proteins of 16 and 110 kDa which appeared at the same isoelectric point on the 2-D gel were identified as the MrgA protein in its monomeric and oligomeric complex forms (15) because both spots were eliminated from 2-D gels of the *mrgA* mutant strain HB1022 (data not shown). The MrgA proteins were induced in the *ahpC* mutant too but at a lower rate than in the wild type. In contrast to the results of Dowds (19), we were not able to detect on the 2-D gels an increased synthesis of MrgA after heat stress (data not shown).

Moreover, proteins of 50 and 41 kDa (Hpi2 and Hpi3) were found to be induced by H_2O_2 in the wild type but not in the mutant. A comparison of other proteins revealed that flagellin

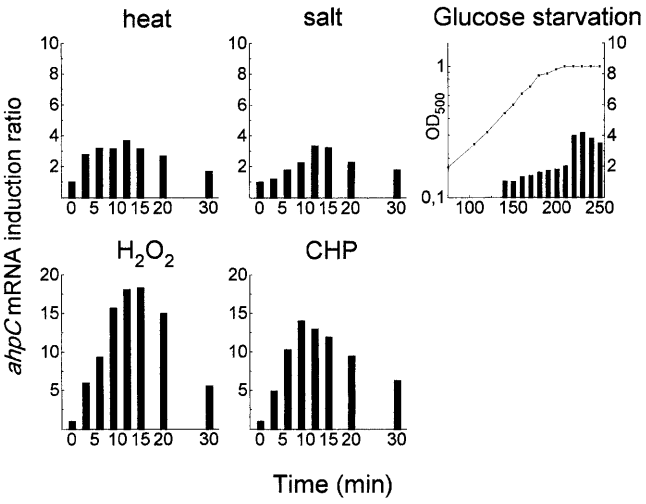

FIG. 2. Quantification of the increase in the amount of *ahpC* mRNA in response to different stresses. Slot blot analysis with RNA isolated from *B. subtilis* IS58 before (0) and at different times after (3, 6, 9, 12, 15, 20, and 30 min) the stress was performed as described in Material and Methods.

(4) was synthesized at a significantly higher rate in the mutant than in the wild type.

Analysis of transcription of the *ahp* operon after stress. Slot blot RNA analysis using an *ahpC*-specific digoxigenin-labelled RNA probe showed a weak but significant three- to fourfold induction of *ahp*-specific mRNA in response to heat and salt stress or after starvation for glucose (Fig. 2). A very strong increase in the amount of the *ahp* mRNA was detected after treatment with 60 μM H_2O_2 (20-fold) or 100 μM CHP (15-fold), which is consistent with the strong induction of AhpC and AhpF on 2-D gels after oxidative stress.

RNA analysis using RNA of the σ^B deletion mutant BGH1 showed the same induction ratios of *ahpC* after stress as the wild-type strain (data not shown).

Northern blot analysis using an *ahpC*-specific mRNA probe was performed to determine the size of the *ahp*-specific transcript. As shown in Fig. 3B, one major transcript of nearly 2.4-kb was detected, which could indicate that *ahpC* and *ahpF* are transcribed in a bicistronic operon from a promoter located downstream from the terminator of the *gnt* operon. The short transcript (Fig. 3B) might be due to degradation.

By primer extension analysis, the 5′ end of the *ahp*-specific transcript was mapped at an adenine residue located 64 nucleotides upstream of the translational start site (Fig. 3A). The transcription was increased weakly in response to heat or salt stress and strongly after exposure to oxidative stress, even in a σ^B mutant. The transcriptional start point is preceded by the sequence TTGACA(X_{17})AATAAT, which perfectly matches all bases of the −35 consensus sequence and five of six bases of the −10 consensus sequence of σ^A-dependent promoters of *B. subtilis*. Sequence analysis showed that the promoter region contains an inverted repeat overlapping the −10 region (Fig. 3C) which demonstrated sequence similarity to the per box consensus (11, 17).

Electron microscopic studies of *ahpC* mutant cells. The *ahpC* mutant was analyzed in exponential and stationary growth phases by electron microscopy. The cells of the wild type grew as long, nonflagellated rods in the exponential growth phase and were smaller, rod shaped, and flagellated after entry into the stationary growth phase. The *ahpC* mutant

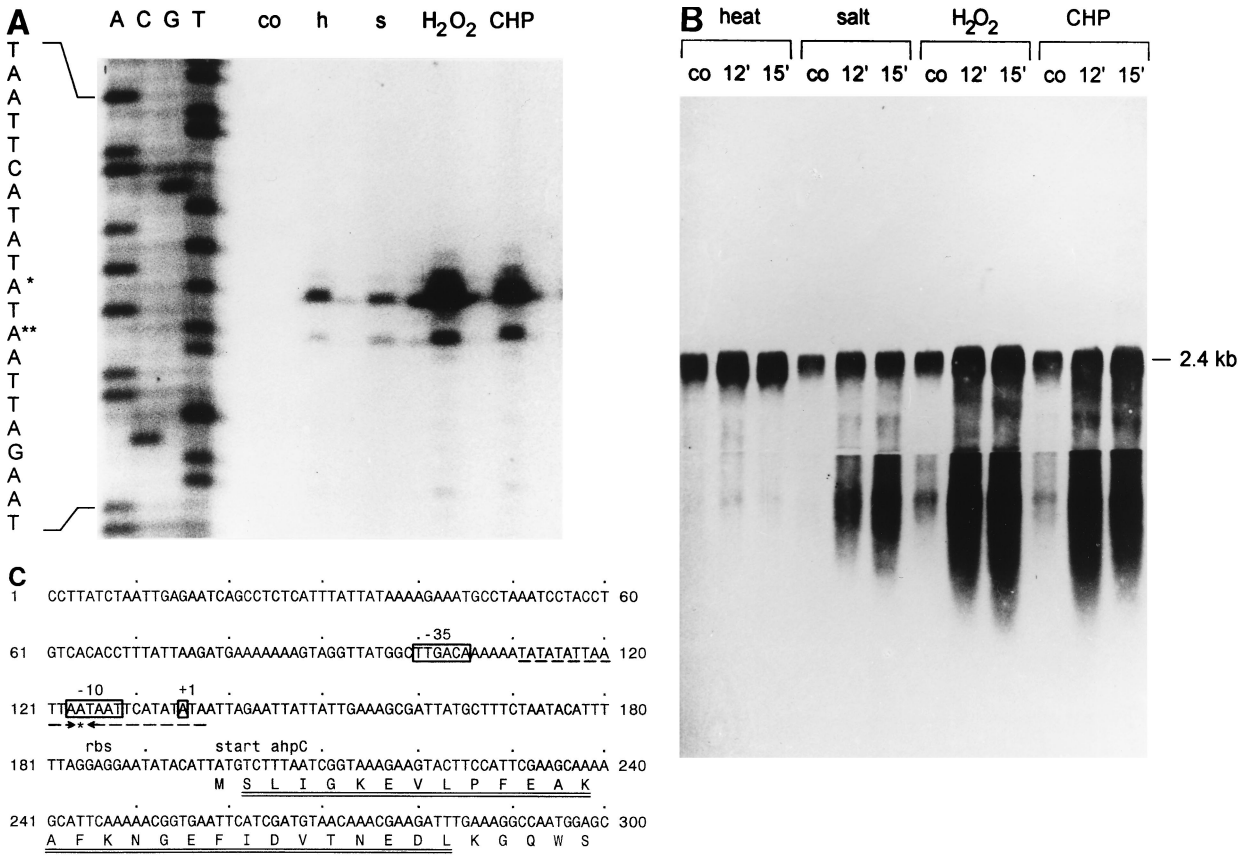

FIG. 3. Transcript analyses of *ahp* after exposure to heat, salt, and oxidative stress and nucleotide sequence of the promoter region. (A) For primer extension analysis, RNA was isolated from *B. subtilis* IS58 before (co) and 12 min after heat shock (50°C) (h), salt stress (4%) (s), or oxidative stress caused by 60 μM H_2O_2 or 100 μM CHP. The potential transcription start site is marked by an asterisk. The dideoxy sequencing ladder (ACGT) extends from the same primer used for primer extension. The sequence displayed is complementary to that determined by DNA sequencing. (B) Northern blot analyses were performed by use of the above-mentioned RNAs isolated before (co) and 12 and 15 min after different stresses were applied. Equal amounts of total RNA (5 μg) were used for primer extension and Northern blot analyses. For technical reasons the Northern blot was cut into two segments, which were hybridized and subjected to autoradiography together. (C) The potential −35 and −10 promoter regions and the start point of transcription (+1) of *ahp* are boxed. The inverted repeat (per box) is indicated by the arrows facing each other below the nucleotide sequence, and the putative Shine-Dalgarno sequence (rbs) is also indicated. The doubly underlined amino acid sequence is identical to that determined from the isolated AhpC protein by N-terminal sequencing.

cells appeared as flagellated rods in both the exponential and stationary growth phases (data not shown).

Ahp and σ^B-dependent GSPs confer resistance to CHP. Deletion of *ahp* in *E. coli* and *S. typhimurium* resulted in hypersensitivity to killing by CHP (53). Furthermore, it has been shown that *ahp* from *S. typhimurium* is involved in the protection of DNA against oxidative damage (34). Therefore, we investigated the oxidative-stress resistance of the *B. subtilis ahpC* mutant strain. Pretreatment of *B. subtilis* IS58 wild-type cells with sublethal doses of H_2O_2 (60 μM) caused the induction of the scavenging enzymes catalase and alkyl hydroperoxide reductase and a DNA-binding and protecting protein (MrgA) (19, 44), which provide specific protection against otherwise lethal H_2O_2 doses (10 mM). Contrary to what was expected, the nonpretreated *ahpC* mutant strain displayed a >100-fold-increased resistance to lethal H_2O_2 challenge in comparison to the wild-type strain. This resistance of the mutant to H_2O_2 could be further increased four to five times if cells were pretreated with 60 μM H_2O_2 prior to the lethal H_2O_2 challenge (Fig. 4A).

Analysis of the rate of survival after treatment of *B. subtilis* cells with different concentrations of CHP in the exponential growth phase revealed that the *ahpC* mutant was 20- to 50-fold more sensitive to lethal levels of CHP (0.5 to 1 mM) than the wild type (Fig. 4B). However, no difference was found for stationary-phase wild-type and mutant cells, which both were completely resistant to 1 mM CHP (Fig. 5).

Since σ^B-dependent GSPs are induced upon entry of cells into stationary phase, we measured the viability of two σ^B deletion mutants (BGH1 and ML6) and their isogenic wild-types parents (IS58 and 168) after treatment with 1 mM CHP at different time points along the growth curve (Fig. 5). The lack of σ^B-dependent GSPs during growth potentiates the killing after CHP challenge more than 100-fold. Stationary-phase σ^B mutant cells displayed an increased resistance to CHP compared with growing cells, but the rate of survival was lower than 10%, whereas wild-type cells showed 100% survival.

DISCUSSION

The induction of GSPs in *B. subtilis* represents one of the first responses of the cell to growth-limiting environmental changes. Besides the large group of σ^B-dependent GSPs, only a few proteins remained stress inducible in a σ^B deletion mutant (class III), exemplified by ClpP, ClpC, ClpX, TrxA, Lon, and FtsH (18, 26, 27, 30, 37, 47, 50). The class III heat shock genes vary in their stress induction pattern, indicating that they may be regulated by different mechanisms (30). The ATP-

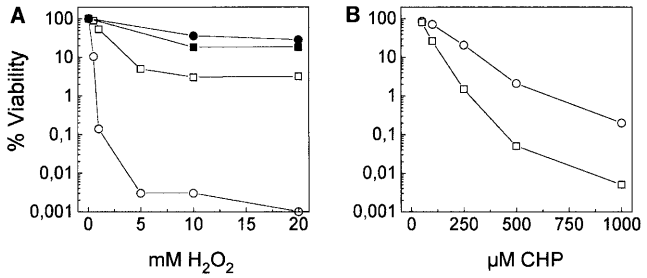

FIG. 4. An *ahpC* mutant strain is resistant to H_2O_2 (A) and sensitive to CHP (B) in the exponential growth phase. (A) Cultures grown in minimal medium to an optical density at 500 nm of 0.4 were divided and either pretreated with 60 μM H_2O_2 for 15 min or not pretreated prior to challenge with higher concentrations of H_2O_2 for 15 min. Strains: IS58, pretreated (●) and nonpretreated (○); BAhpC, pretreated (■) and nonpretreated (□). (B) Cultures grown to an optical density at 500 nm of 0.4 were treated with various concentrations of CHP for 15 min. Strains: IS58 (○) and BAhpC (□). Viability was determined by measuring CFUs. The values are the averages of the results obtained in five independent experiments for each strain.

dependent chaperones and proteases ClpC and ClpP as well as TrxA are strongly induced by a σ^B-dependent as well as a σ^B-independent mechanism (27, 36, 50). In this study, a new class III heat shock protein was identified as AhpC, the small subunit of the alkyl hydroperoxide reductase which is induced weakly by heat or salt stress by a strictly σ^B-independent mechanism. This induction of *ahp* in response to heat or salt stress resembles the induction of three other class III genes, *lon*, *clpX*, and *ftsH* (18, 26, 47). They are preceded by putative σ^A-dependent promoters which are induced three- to fivefold in response to heat or salt stress. However, in contrast to other class III genes, the *ahp* operon is strongly induced by oxidative stress.

Per boxes which overlap with the *ahpC* promoter region may be the target sites for the putative peroxide regulon repressor (17). Similar operator sites exist in the promoter regions of *katA*, *mrgA*, and *hemA* (8, 16, 17, 46). Therefore, it seems likely that the *ahp* operon is regulated in the same way as *katA*, *mrgA*, or the *hem* operon in response to oxidative stress (17). The finding that the *ahp* operon can be induced by heat or salt stress whereas *katA* (8, 23) and *mrgA* are only induced by oxidative stress indicates that an additional *cis*-acting element in the regulatory region of *ahp* might be responsible for the induction by heat or salt stress.

Hartford and Dowds (28) isolated a spontaneous H_2O_2-resistant mutant, MA991, which overproduces all proteins of the peroxide regulon, including AhpC and AhpF. Two features of this mutant, repression of flagellin and filamentous growth and increased resistance to organic hydroperoxides (28), might be related to alterations in the levels of AhpC and AhpF.

2-D gel analyses revealed that flagellin was overproduced in *ahpC* mutant cells, resulting in a constitutive flagellated phenotype. This result indicates that flagellin synthesis may be repressed when AhpC and AhpF are overproduced either in the mutant or in response to oxidative stress.

An *ahpC* mutant was more sensitive to CHP than the wild type during exponential growth. Therefore, we propose that the alkyl hydroperoxide reductase of *B. subtilis* is involved in detoxification of organic hydroperoxides which are produced from unsaturated fatty acids and nucleic acids during oxidative stress conditions in growing cells.

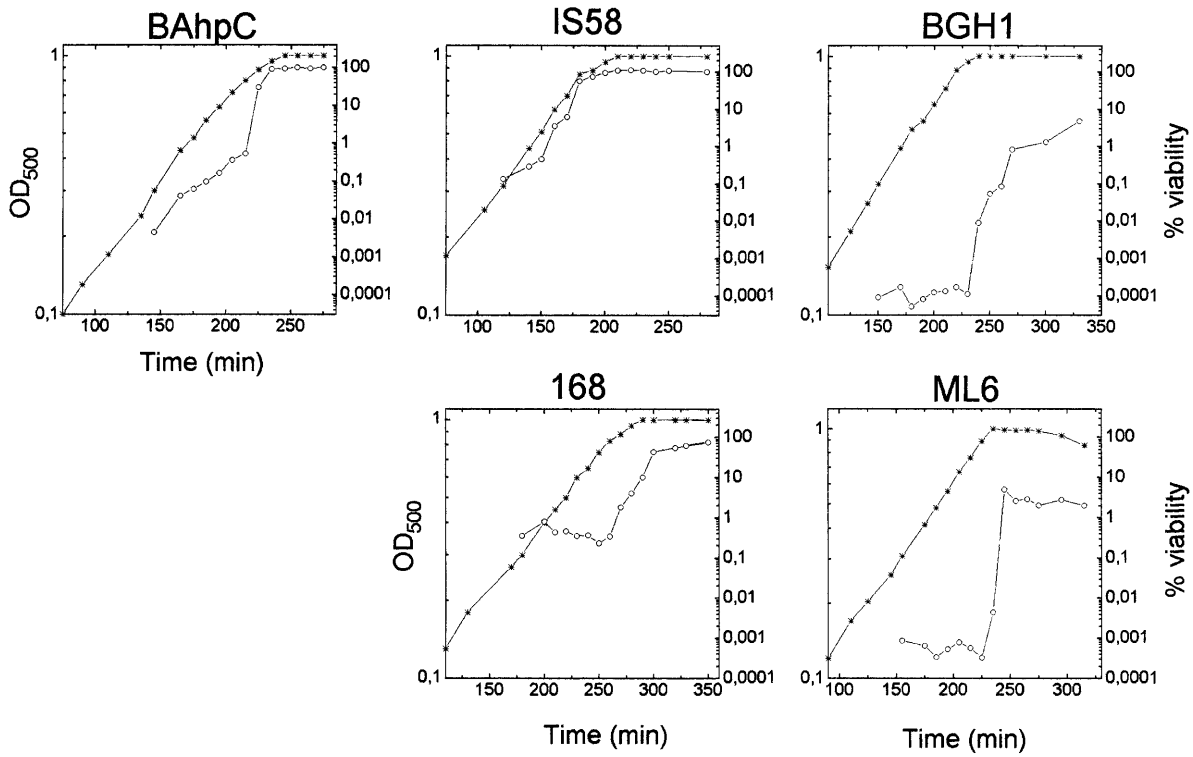

FIG. 5. Growth stage-dependent sensitivity to CHP. The strains used were wild-type IS58, the isogenic *ahpC* mutant BAhpC and σ^B mutant BGH1, wild-type 168, and the isogenic σ^B mutant ML6. Symbols: ∗, culture density; ○, percent viability after CHP challenge. Cultures grown in minimal medium under glucose starvation conditions were exposed to 1 mM CHP for 15 min at the time points indicated and then plated for survivors. The values are the averages of the results obtained in five independent determinations.

Interestingly, both MA991 (28) and our *ahpC* mutant displayed a constitutive hydrogen peroxide-resistant phenotype. As reported in the accompanying paper (11), the increased resistance of *ahpC* mutant cells to H_2O_2 may be due to the derepression of *katA* and other peroxide genes. This is consistent with the results of our 2-D gel analyses of oxidatively stressed wild-type and mutant cells, which revealed an increased basal level of KatA in the *ahpC* mutant. The resistance of the mutant to 10 mM H_2O_2 could be further increased by preadaptation, which might be due to the induction of KatA and MrgA, as visualized on the 2-D gels. To confirm that the H_2O_2-resistant phenotype was associated with an *ahp* allele, a shuttle vector containing the *ahp* operon was transformed into the *ahpC* mutant strain, which restored the H_2O_2 sensitivity of the wild type (data not shown).

The *ahp* operon was also induced after starvation for glucose. However, in stationary-phase cells, Ahp is not required to confer resistance to CHP because stationary-phase wild-type and *ahpC* mutant cells are both resistant to CHP. A second mechanism must be responsible for this resistance. Surprisingly, σ^B mutants are characterized by a dramatically enhanced sensitivity to CHP. In the literature, there is no evidence that the phenotype of σ^B mutants is altered compared with that of the wild type, which makes the prediction of the functions of σ^B-dependent proteins questionable (7, 21). However, Engelmann et al. (23) found that σ^B controls the expression of *katE* of *B. subtilis*, which is highly homologous to the starvation-induced σ^S-dependent catalase gene *katE* from *E. coli*. σ^S is a master regulator of *E. coli* that is involved in multiple-stress resistance in starved cells (39). Therefore, it was tempting to speculate that σ^B might provide a similar unspecific protective function for *B. subtilis* (9, 23, 30). Recent studies by Engelmann and Hecker (22) and our results seem to confirm this prediction, at least for the oxidative-stress resistance. However, some further resistance to CHP could also be induced in glucose-starved σ^B mutant cells, indicating that another σ^B-independent system exists which may cause the increased stationary-phase resistance to CHP.

In summary, the expression, regulation, and function of *ahpCF* during oxidative stress are similar to those of the *oxyR*-dependent *ahp* operon of *E. coli*. Because of its σ^B-independent induction in response to other stresses and starvation, it belongs in GSP class III. Phenotypic studies of *ahpC* and σ^B mutants have shown that both oxidative and general stress responses are involved in the protection of the cell against the toxic oxidant CHP.

ACKNOWLEDGMENTS

We are very grateful to John Helmann for communication of unpublished results, comments on the manuscript, and providing strain HB1022; N. Ogasawara for sharing sequencing results; and Richard Losick for providing strain ML6. Furthermore, we thank Uwe Völker and Gerhard Mittenhuber for critical reading of the manuscript, Renate Hanschke for help with electron microscopy, and Hiltraud Mach for performance of analytical 2-D gels. We gratefully acknowledge Eva Uhlemann, Anita Harang, Renate Gloger, and Karin Binder for assistance with some experiments and Gertrud Schitteck and Sabine Schade for preparing the photographs.

This work was supported by grants from BMBF, DFG, and the Fonds der Chemischen Industrie to M.H. R.S. was supported by DFG grants to SFB171/B4, Universität Osnabrück, where protein sequencing was carried out.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Anagnostopoulos, C., P. J. Piggot, and J. A. Hoch.** 1993. The genetic map of Bacillus subtilis, p. 425–461. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. American Society for Microbiology, Washington, D.C.
3. **Anagnostopoulos, C., and J. Spizizen.** 1961. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol. **81:**741–746.
4. **Antelmann, H., J. Bernhardt, R. Schmid, and M. Hecker.** 1995. A gene at 333° on the *Bacillus subtilis* chromosome encodes the newly identified σ^B-dependent general stress protein GspA. J. Bacteriol. **177:**3540–3545.
5. **Armstrong-Buisseret, L., M. B. Cole, and G. S. A. B. Stewart.** 1995. A homologue to the *Escherichia coli* alkyl hydroperoxide reductase AhpC is induced by osmotic upshock in *Staphylococcus aureus*. Microbiology **141:** 1655–1661.
6. **Bernhardt, J., U. Völker, A. Völker, H. Antelmann, R. Schmid, H. Mach, and M. Hecker.** Specific and general stress proteins in *Bacillus subtilis*—a two-dimensional electrophoretical study. Submitted for publication.
7. **Binnie, C., M. Lampe, and R. Losick.** 1986. Gene encoding the σ^{37} species of RNA polymerase factor from *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **83:**5943–5947.
8. **Bol, D. K., and R. E. Yasbin.** 1991. The isolation, cloning and identification of a vegetative catalase gene from *Bacillus subtilis*. Gene **109:**31–37.
9. **Boylan, S. A., A. R. Redfield, M. S. Brody, and C. W. Price.** 1993. Stress-induced activation of the σ^B transcription factor of *Bacillus subtilis*. J. Bacteriol. **175:**7931–7937.
10. **Boylan, S. A., A. R. Redfield, and C. W. Price.** 1993. Transcription factor σ^B of *Bacillus subtilis* controls a large stationary-phase regulon. J. Bacteriol. **175:**3957–3963.
11. **Bsat, N., L. Chen, and J. D. Helmann.** 1996. Mutation of the *Bacillus subtilis* alkyl hydroperoxide reductase (*ahpCF*) operon reveals compensatory interactions among hydrogen peroxide stress genes. J. Bacteriol. **178:**6579–6586.
12. **Chae, H. Z., S. J. Chung, and S. G. Rhee.** 1994. Thioredoxin-dependent peroxide reductase from yeast. J. Biol. Chem. **269:**27670–27678.
13. **Chae, H. Z., K. Robison, L. B. Poole, G. Church, G. Storz, and S. G. Rhee.** 1994. Cloning and sequencing of thiol-specific antioxidant from mammalian brain: alkyl hydroperoxide reductase and thiol-specific antioxidant proteins define a large family of antioxidant enzymes. Proc. Natl. Acad. Sci. USA **91:**7017–7021.
14. **Chae, H. Z., T. B. Uhm, and S. G. Rhee.** 1994. Dimerization of thiol-specific antioxidant and the essential role of cysteine 47. Proc. Natl. Acad. Sci. USA **91:**7022–7026.
15. **Chen, L., and J. D. Helmann.** 1995. *Bacillus subtilis* MrgA is a Dps (PexB) homologue: evidence for metalloregulation of an oxidative-stress gene. Mol. Microbiol. **18:**295–300.
16. **Chen, L., L. P. James, and J. D. Helmann.** 1993. Metalloregulation in *Bacillus subtilis*: isolation and characterization of two genes differentially repressed by metal ions. J. Bacteriol. **175:**5428–5437.
17. **Chen, L., L. Keramati, and J. D. Helmann.** 1995. Coordinate regulation of *Bacillus subtilis* peroxide stress genes by hydrogen peroxide and metal ions. Proc. Natl. Acad. Sci. USA **92:**8190–8194.
18. **Deuerling, E., B. Paeslack, and W. Schumann.** 1995. The *ftsH* gene of *Bacillus subtilis* is transiently induced after osmotic and temperature upshift. J. Bacteriol. **177:**4105–4112.
19. **Dowds, B. C. A.** 1994. The oxidative stress response in *Bacillus subtilis*. FEMS Microbiol. Lett. **124:**255–264.
20. **Dowds, B. C. A., P. Murphy, D. J. McConnell, and K. M. Devine.** 1987. Relationship among oxidative stress, growth cycle, and sporulation in *Bacillus subtilis*. J. Bacteriol. **169:**5771–5775.
21. **Duncan, M. L., S. S. Kalman, S. M. Thomas, and C. W. Price.** 1987. Gene encoding the 37,000-dalton minor sigma factor of *Bacillus subtilis* RNA polymerase: isolation, nucleotide sequence, chromosomal location, and cryptic function. J. Bacteriol. **169:**771–778.
22. **Engelmann, S., and M. Hecker.** Impaired oxidative stress resistance of Bacillus subtilis sigB mutants and the role of katA and katE, FEMS Microbiol. Lett., in press.
23. **Engelmann, S., C. Lindner, and M. Hecker.** 1995. Cloning, nucleotide sequence, and regulation of *katE* encoding a σ^B-dependent catalase in *Bacillus subtilis*. J. Bacteriol. **177:**5598–5605.
24. **Ferrari, F. A., A. Nguyen, D. Lang, and J. D. Hoch.** 1983. Construction and properties of an integrable plasmid for *Bacillus subtilis*. J. Bacteriol. **154:** 1513–1515.
25. **Fujita, Y., T. Fujita, Y. Miwa, J. Nihashi, and Y. Aratani.** 1986. Organization and transcription of the gluconate operon, *gnt*, of *Bacillus subtilis*. J. Biol. Chem. **261:**13744–13753.
26. **Gerth, U., N. Carter, P. Emmerson, C. R. Harwood, N. Wipat, and M. Hecker.** Sequence and transcriptional analysis of *Bacillus subtilis clpX*—a class III heat shock gene. Gene, in press.
27. **Gerth, U., and M. Hecker.** Unpublished data.
28. **Hartford, O. M., and B. C. A. Dowds.** 1994. Isolation and characterization of a hydrogen peroxide resistant mutant of *Bacillus subtilis*. Microbiology **140:** 297–304.
29. **Hecker, M., C. Heim, U. Völker, and L. Wölfel.** 1988. Induction of stress

proteins by sodium chloride treatment in *Bacillus subtilis*. Arch. Microbiol. **150:**564–566.
30. **Hecker, M., W. Schumann, and U. Völker.** 1996. Heat-shock and general stress response in *Bacillus subtilis*. Mol. Microbiol. **19:**417–428.
31. **Hecker, M., and U. Völker.** 1990. General stress proteins in *Bacillus subtilis*. FEMS Microbiol. Ecol. **74:**197–213.
32. **Hoch, J. A.** 1991. Genetic analysis in *Bacillus subtilis*. Methods Enzymol. **204:**305–320.
33. **Igo, M., M. Lampe, C. Ray, W. Schafer, C. P. Moran, Jr., and R. Losick.** 1987. Genetic studies of a secondary RNA polymerase sigma factor in *Bacillus subtilis*. J. Bacteriol. **169:**3464–3469.
34. **Jacobson, F. S., R. W. Morgan, M. F. Christman, and B. N. Ames.** 1989. An alkyl hydroperoxide reductase from *Salmonella typhimurium* involved in the defense of DNA against oxidative damage. J. Biol. Chem. **264:**1488–1496.
35. **Kasahara, Y., S. Nakai, H. Yoshikawa, and N. Ogasawara.** 1997. Unpublished data.
36. **Krüger, E., T. Msadek, and M. Hecker.** 1996. Alternate promoters direct stress-induced transcription of the *Bacillus subtilis clpC* operon. Mol. Microbiol. **20:**713–723.
37. **Krüger, E., U. Völker, and M. Hecker.** 1994. Stress induction of *clpC* in *Bacillus subtilis* and its involvement in stress tolerance. J. Bacteriol. **176:** 3360–3367.
38. **Li, M., and S.-L. Wong.** 1992. Cloning and characterization of the *groESL* operon from *Bacillus subtilis*. J. Bacteriol. **174:**3981–3992.
39. **Loewen, P. C., and R. Hengge-Aronis.** 1994. The role of the sigma factor σ^S (katF) in bacterial global regulation. Annu. Rev. Microbiol. **48:**53–80.
40. **Majumdar, D., Y. J. Avissar, and J. H. Wyche.** 1991. Simultaneous and rapid isolation of bacterial and eukaryotic DNA and RNA—a new approach for isolating DNA. BioTechniques **11:**94–101.
41. **Mathieu, I., J. Meyer, and J.-M. Moulis.** 1992. Cloning, sequencing and expression in *Escherichia coli* of the rubredoxin gene from *Clostridium pasteurianum*. J. Biochem. **285:**255–262.
42. **Maul, B., U. Völker, S. Riethdorf, S. Engelmann, and M. Hecker.** 1995. SigB-dependent induction of *gsiB* by multiple stimuli in *Bacillus subtilis*. Mol. Gen. Genet. **248:**114–120.
43. **Meade, H. M., S. R. Long, G. B. Ruvkun, S. E. Brown, and F. E. Ausubel.** 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by Tn*5* mutagenesis. J. Bacteriol. **149:**114–122.
44. **Murphy, P., B. C. A. Dowds, D. J. McConnell, and K. M. Devine.** 1987. Oxidative stress and growth temperature in *Bacillus subtilis*. J. Bacteriol. **169:**5766–5770.
45. **Niimura, Y., K. Ohnishi, Y. Yarita, M. Hidaka, H. Masaki, T. Uchimura, H. Suzuki, M. Kozaki, and T. Uozumi.** 1993. A flavoprotein functional as NADH oxidase from *Amphibacillus xylanus* Ep01: purification and characterization of the enzyme and structural analysis of its gene. J. Bacteriol. **175:**7945–7950.
46. **Petricek, M., L. Rutberg, I. Schröder, and L. Hederstedt.** 1990. Cloning and characterization of the *hemA* region of the *Bacillus subtilis* chromosome. J. Bacteriol. **172:**2250–2258.
47. **Riethdorf, S., U. Völker, U. Gerth, A. Winkler, S. Engelmann, and M. Hecker.** 1994. Cloning, nucleotide sequence, and expression of the *Bacillus subtilis lon* gene. J. Bacteriol. **176:**6518–6527.
48. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
49. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
50. **Scharf, C., S. Riethdorf, H. Ernst, S. Engelmann, U. Völker, and M. Hecker.** Thioredoxin is an essential protein induced by heat and other stresses in Bacillus subtilis. Submitted for publication.
51. **Schmidt, A., M. Schiesswohl, U. Völker, M. Hecker, and W. Schumann.** 1992. Cloning, sequencing, mapping, and transcriptional analysis of the *groESL* operon from *Bacillus subtilis*. J. Bacteriol. **174:**3993–3999.
52. **Smith, I., P. Paress, K. Cabane, and E. Dubnau.** 1980. Genetics and physiology of the *rel* system of *Bacillus subtilis*. Mol. Gen. Genet. **178:**271–279.
53. **Storz, G., F. S. Jacobson, L. A. Tartaglia, R. W. Morgan, L. A. Silveira, and B. N. Ames.** 1989. An alkyl hydroperoxide reductase induced by oxidative stress in *Salmonella typhimurium* and *Escherichia coli*: genetic characterization and cloning of *ahp*. J. Bacteriol. **171:**2049–2055.
54. **Stülke, J., R. Hanschke, and M. Hecker.** 1993. Temporal activation of β-glucanase synthesis in *Bacillus subtilis* is mediated by the GTP pool. J. Gen. Microbiol. **39:**2041–2045.
55. **Tai, S. S., and Y. Y. Zhu.** 1995. Cloning of a *Corynebacterium diphtheriae* iron-repressible gene that shares sequence homology with the AhpC subunit of alkyl hydroperoxide reductase of *Salmonella typhimurium*. J. Bacteriol. **177:**3512–3517.
56. **Tartaglia, L. A., G. Storz, M. H. Brodsky, A. Lai, and B. N. Ames.** 1990. Alkyl hydroperoxide reductase from *Salmonella typhimurium*. J. Biol. Chem. **265:** 10535–10540.
57. **Völker, U., S. Engelmann, B. Maul, S. Riethdorf, A. Völker, R. Schmid, H. Mach, and M. Hecker.** 1994. Analysis of the induction of general stress proteins of *Bacillus subtilis*. Microbiology **140:**741–752.
58. **Wada, K., Y. Wada, H. Doi, F. Ishibashi, T. Gojobori, and T. Ikemura.** 1991. Codon usage tabulated from the GenBank genetic sequence data. Nucleic Acids Res. **19:**1981–1986.
59. **Wetzstein, M., U. Völker, J. Dedio, S. Löbau, U. Zuber, M. Schiesswohl, C. Herget, M. Hecker, and W. Schumann.** 1992. Cloning, sequencing, and molecular analysis of the *dnaK* locus from *Bacillus subtilis*. J. Bacteriol. **174:** 3300–3310.
60. **Xu, X., N. Koyama, M. Cui, A. Yamagishi, Y. Nosoh, and T. Oshima.** 1991. Nucleotide sequence of the gene encoding NADH dehydrogenase from an alcalophile, *Bacillus* sp. strain YN-1. J. Biochem. **109:**678–683.
61. **Yuan, G., and S.-L. Wong.** 1995. Regulation of *groE* expression in *Bacillus subtilis*: the involvement of the σ^A-like promoter and the roles of the inverted repeat sequence (CIRCE). J. Bacteriol. **177:**5427–5433.
62. **Yuan, G., and S.-L. Wong.** 1995. Isolation and characterization of *Bacillus subtilis groE* regulatory mutants: evidence for *orf39* in the *dnaK* operon as a repressor gene in regulating the expression of both *groE* and *dnaK*. J. Bacteriol. **177:**6462–6468.
63. **Zhang, J., and A. Aronson.** 1994. A *Bacillus subtilis bglA* gene encoding phospho-β-glucosidase is inducible and closely linked to an NADH dehydrogenase-encoding gene. Gene **140:**85–90.
64. **Zuber, U., and W. Schumann.** 1994. CIRCE, a novel heat shock element involved in regulation of heat shock operon *dnaK* of *Bacillus subtilis*. J. Bacteriol. **176:**1359–1363.