Studies on Specific and General Defense Strategies against Reactive Oxygen Species in *Bacillus subtilis*

I n a u g u r a l d i s s e r t a t i o n

zur

Erlangung des akademischen Grades

doctor rerum naturalium (Dr. rer. nat.)

an der Mathematisch-Naturwissenschaftlichen Fakultät

der

Ernst-Moritz-Arndt-Universität Greifswald

vorgelegt von

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geboren am 31.07.1971

in Berlin

Greifswald, 02.02.2004

Dekan:

1. Gutachter:
2. Gutachter:

Tag der Promotion:

Contents

Summary

The present work consists of four parts, containing experimental data obtained from analysis of *Bacillus subtilis* specific and general defense strategies against reactive oxygen species.

In the first part, the peroxide and superoxide stress stimulons of *B. subtilis* were analyzed by means of transcriptomics and proteomics. Oxidative stress responsive genes were classified into two groups: the gene expression pattern was either similar after both stresses or the genes primarily responded to one stimulus. The high induction observed for members of the PerR-regulon after both stimuli supported the assumption that activation of the peroxide specific PerR-regulon represented the primary stress response after superoxide and peroxide stress.[1]

The second part focuses on protein carbonylation in *B. subtilis* wild-type and *sigB* mutant cells. The introduction of carbonyl groups into amino acid side chains of proteins represents one possible form of protein modification after attack by reactive oxygen species. Carbonyl groups are readily detectable and the observed amounts can thus serve as an indicator for the severity of protein damage. The results demonstrate clearly that *B. subtilis* proteins are susceptible to hydrogen peroxide (H_2O_2) mediated carbonylation damage. The application of low concentrations of H_2O_2 prior to the exposure to otherwise lethal levels of peroxide reduced markedly the degree of protein carbonylation, which also held true for glucose starved cells. Artificial preloading with general stress proteins resulted in a lower level of protein carbonylation when cells were subjected to oxidative stress, but no differences were detected between wild-type and *sigB* mutant cells.

In the third part, strains with mutations in genes encoding general stress proteins were screened for decreased resistance after H_2O_2 challenge. It was demonstrated that resistance to H_2O_2 after transient heat treatment, likewise to conditions of glucose starvation, was at least partly mediated by the σ^B-dependent general stress response. The screening of mutants in σ^B-controlled genes revealed an important role for the deoxyribonucleic acid (DNA)-binding protein Dps in the context of σ^B-mediated resistance to oxidative stress underlining previous reports. Therefore, the experimental strategy opens a global view on the importance of DNA integrity in *B. subtilis* under conditions of oxidative stress.

The fourth part includes analysis of a *B. subtilis* thioredoxin conditional mutant. The thiol-disulfide oxidoreductase TrxA is an essential protein in *B. subtilis* that is suggested to be involved in maintaining the cytoplasmic thiol-disulfide state even under conditions of oxidative stress. To investigate the physiological role of TrxA, growth experiments and two-dimensional gel electrophoresis were carried out with exponentially growing cells that were depleted of TrxA. The observations indicate that TrxA is essentially involved in the re-reduction of phosphoadenosyl phosphosulfate reductase CysH within the sulfate assimilation pathway of *B. subtilis*.

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[1] This part was done in cooperation with C. Scharf. Both authors contributed equally to it.

Zusammenfassung

Die vorliegende Arbeit, bestehend aus vier Abschnitten, enthält experimentelle Daten aus Analysen der spezifischen und generellen Schutzstrategien von *Bacillus subtilis* gegen reaktive Sauerstoffspezies.

In dem ersten Abschnitt ist die Analyse der Peroxid und Superoxid Stressstimulons von *B. subtilis* anhand von Transkriptom- und Proteomdaten dargestellt. Gene, die auf oxidativen Stress reagieren, konnten zwei Gruppen zugeordnet werden: entweder waren die Genexpressionsmuster nach Einwirkung beider Stressoren ähnlich, oder die Genexpression wurde nur durch einen der beiden Stimuli verändert. Die starke Induktion, die für Gene des PerR-Regulons nach beiden Stimuli beobachtet wurde, unterstützt die These, dass die Aktivierung des Peroxid-spezifischen PerR-Regulons die primäre Stressantwort nach Superoxid- und nach Peroxidstress darstellt.[2]

Der zweite Abschnitt enthält Daten zu Proteincarbonylierungen in Wildtyp- und *sigB* Mutantenzellen von *B. subtilis*. Die Reaktion von Aminosäureseitenketten zu Carbonyl-haltigen Derivaten stellt eine der möglichen Formen von Proteinmodifikationen nach Oxidation durch reaktive Sauerstoffspezies dar. Der Nachweis von Proteincarbonylierungen ist methodisch gut zugänglich und ermittelte Mengen können als Indikatoren für das Level der oxidativen Proteinschädigung herangezogen werden. Die Resultate belegen, dass Proteine von *B. subtilis* nach Einwirkung von Hydrogenperoxid (H_2O_2) Carbonylierungsschäden aufweisen. Der Grad der Schädigung war weniger ausgeprägt, wenn Zellen vor einer lethalen Peroxidbehandlung mit niedrigen Konzentrationen von H_2O_2 vorbehandelt wurden oder einer zeitgleichen Glukoselimitation ausgesetzt waren. Nach künstlicher Beladung mit Generellen Stress Proteinen konnte ebenso eine erniedrigte Menge von Carbonylgruppen nach oxidativem Stress beobachtet werden, jedoch wurden keine Unterschiede zwischen Wildtyp- und Δ*sigB*-Zellen festgestellt.

Im dritten Abschnitt wird die Analyse von Stämmen mit Mutationen in Sigmafaktor σ^B-regulierten Genen im Hinblick auf verringerte Resistenz gegenüber H_2O_2 beschrieben. Es konnte gezeigt werden, dass eine erhöhte Resistenz gegenüber H_2O_2 nach einer kurzzeitigen Temperaturerhöhung, ähnlich einer Glukoselimitation, zumindest teilweise auf der σ^B-vermittelten Generellen Stressantwort beruht. Durch die Analyse von Mutanten in σ^B-kontrollierten Genen konnte für das Desoxyribonukleinsäure-(DNS)-bindende Protein Dps eine wichtige Rolle innerhalb der σ^B-vermittelten Resistenz gegenüber H_2O_2 ermittelt werden, womit diese Resultate frühere Beobachtungen unterstützen. Die experimentelle Strategie eröffnet einen globalen Blick auf die Wichtigkeit der DNS-Integrität in *B. subtilis* nach oxidativem Stress.

Der vierte Abschnitt enthält Analysen einer *B. subtilis* Thioredoxin Konditionalmutante. Die Thiol-Disulfid Oxidoreduktase TrxA ist ein essentielles Protein in *B. subtilis*, das vermutlich in die Aufrechterhaltung des zytoplasmatischen Thiol-Disulfid-Status auch unter oxidativen Stressbedingungen involviert ist. Die Untersuchung der physiologischen Funktion von TrxA wurde mittels Wachstumsexperimenten und zwei-dimensionaler Gel Elektrophorese an Zellen durchgeführt, die kein TrxA Protein enthielten. Die Beobachtungen weisen auf eine essentielle Beteiligung von TrxA an der Re-Reduktion der Phosphoadenosyl Phosphosulfat Reduktase CysH innerhalb der Sulfat-Assimilation hin.

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[2] Dieser Abschnitt wurde gemeinsam mit C. Scharf bearbeitet. Beide Autoren trugen gleichteilig dazu bei.

Introduction

Experimental system

Bacillus subtilis. Bacteria of the species *Bacillus subtilis* are members of the genus *Bacillus* that was created in 1872 (Cohn, 1872). Representatives are aerobic, endospore-forming, rod-shaped bacteria with a Gram-positive cell wall structure (Sneath, 1986). *B. subtilis* itself represents the type species of the genus. In addition to this non-pathogenic microorganism, human-pathogenic *B. cereus* and *B. anthracis*, the insect pathogens *B. thuringiensis*, *B. larvae* and *B. popilliae* and thermophilic *B. stearothermophilus* are all included in this heterogeneous group (Sneath, 1986). Altogether, it contains about 50 validly described species. The soil-bacterium *B. subtilis* is an extensively investigated member of the genus *Bacillus* and became the most comprehensively studied bacterium such as the Gram-negative endobacterium *Escherichia coli*.

Cells of *B. subtilis* were originally isolated and described by Ferdinand Cohn (Cohn, 1872). In the following, a strain from the University of Marburg, designated NCTC3610 (National Collection of Type Cultures, London, GB) respectively ATCC6051 (American Type Culture Collection, Rockville, USA), was adopted as the type strain of the species *B. subtilis*, after it was carefully compared to Cohn's original isolate (Conn, 1930). The generation of a large number of auxotrophic mutants led to the specification of a tryptophan-requiring strain, *B. subtilis* strain 168, after the cells of the type strain were exposed to X-ray and UV-light (Burkholder & Giles, 1947). Spizizen demonstrated that this strain was readily transformable and prepared the ground for the adoption of *B. subtilis* 168 to genetic experimentation (Spizizen, 1958; Anagnostopoulos & Spizizen, 1961). The invention of the recombinant deoxyribonucleic acid (DNA) technology and the DNA transformation technique enabled an extensive investigation of the physiology of *B. subtilis* 168. Within an international network approach in the 1990's, the genome sequence of *B. subtilis* strain 168 was determined and the annotated sequence data has been made available (Kunst et al., 1997). Even if the exact origin of *B. subtilis* 168 is not recorded in all details, this strain is considered to be closely related to if not even identical with the type strain NCTC3610 of the species *Bacillus subtilis*.

Adaptive response networks

Natural habitat. *B. subtilis* is a chemoorganotrophic bacterium with the ability to oxidize a variety of organic compounds (Sneath, 1986). It can be isolated from soil, where it is most

active in the aerobic, upper layer. Similar to other soil-living *Bacillus* species, *B. subtilis* can be regarded as r-strategist that shows metabolic activation when nutrients become available (Sneath, 1986). In contrast to the exponential growth observed under laboratory conditions, in its natural ecosystem physical stress and nutrient limitation restrict the growth of populations to slow rates. The permanent variation of biotic and abiotic factors rather enforce the cellular system to focus on repeated adaptation in order to survive under hazardous environmental conditions (Kjelleberg, 1993; Morita, 1997). As a consequence of the effort to adjust the physiological state most economically, *B. subtilis* cells respectively ancestral cells, have developed highly complex response networks during their evolution.

Adaptive networks. Bacterial cells may alter morphology, metabolism and motility in order to adapt to environmental fluctuations. The phenotypical responses are based on highly sophisticated systems to percept environmental signals which subsequently trigger changes in gene expression or protein synthesis. *B. subtilis* represents a group of bacteria with two outstanding adaptive strategies that are activated by nutrient limitations: sporulation and natural competence.

The cessation of growth enforced by starvation or high cell density can lead to the production of dormant endospores which are able to outlast extended periods of nutrient limitation and are highly resistant to various kinds of physical stress. The differentiation of vegetative cells includes a comprehensive change of the global gene expression program and the process is not reversible beyond a certain point after its initiation. If the environmental situation improves, germination and outgrowth of spores to vegetative, reproducing cells will secure the survival of the population (for review see Yudkin, 1993; Sonenshein, 2000a; Sonenshein, 2000b; Piggot & Losick, 2002).

Another complex reprogramming of gene expression can be observed in the process of competence. After stimulation by environmental conditions, *B. subtilis* cells are able to bind extracellular nucleic acids and subsequently internalize the macromolecular DNA. The induction of the competence regulon may contribute to restore damaged DNA and/or may enhance the genetic diversity by the generation of new allelic combinations. This idea is supported by the observation that the competence and SOS-DNA-repair regulons are co-induced (Love et al., 1985). Therefore, the ability of DNA uptake with a subsequent recombination event is suggested to provide selective advantages under conditions of nutrient limitation, finally securing the survival of the population (for review see Dubnau & Turgay, 2000; Dubnau & Lovett, 2002).

Sporulation and competence represent time-consuming adaptive responses that might not be the most efficient strategies in response to each stress condition. Both strategies are only two examples of a number of adaptive strategies that can be activated in a bacterial cell in response to threatening environmental conditions. In fact, under conditions of nutrient limitation only a limited part of a *B. subtilis* population will develop endospores or will become competent. Therefore, the importance of other strategies to confer stress resistance and facilitate survival has to be emphasized. For example, the sigma factor σ^B-dependent general stress response and the RelA-regulated stringent response both represent putative alternatives to sporulation and competence (Hecker & Völker, 2001; Eymann et al., 2002). Furthermore, a set of specific strategies can be activated in bacterial cells in response to their particular stimuli to provide specific stress resistance. All stress response modules of a cell are embedded into the highly complex network of signal perception, signal transduction and activation of gene expression in order to secure the survival of the population.

Specific stress responses. Under laboratory conditions, a number of adaptive strategies has been identified which are activated in response to sublethal changes of one specific stimulus. In general, a specific stimulus induces a set of genes whose products confer protection against higher levels of the same type of stress. For *B. subtilis*, experiments with temperature shifts, changes of osmotic conditions or exposure to oxidative agents as well as investigations of limitations in carbon sources, phosphate or oxygen elucidated specifically induced responses that provide the cells with specific protection against a particular stress condition.

One of the best studied examples in this context is the induction of specific heat shock genes after temperature upshift in *B. subtilis*. Under conditions of 37°C, the regulatory protein HrcA binds to an inverted repeat (CIRCE, *Controlling Inverted Repeat of Chaperone Expression*) in the operator region in front of two target operons (Zuber & Schumann, 1994; Schulz & Schumann, 1996). Consequently, transcriptional initiation is blocked by RNA polymerase containing the vegetative sigma factor σ^A. After temperature upshift, the sudden accumulation of denatured proteins titrates the HrcA-regulated chaperone GroEL/ES that on its part fails to refold HrcA into its active state. In the following, the heat-specific regulon, consisting of two operons, is derepressed and the chaperone complexes GroEL/ES and DnaK are synthesized in high amounts (Mogk et al., 1997; Mogk et al., 1998). If the refolding of denatured proteins was successful that no or

only small amounts of protein aggregates remain in the cytoplasm, unoccupied GroEL/ES will catalyze the transformation of HrcA back into its active state and the transcription of the heat stress specific regulon is discontinued (Mogk et al., 1997).

However, the specific stress response is not the only change of gene expression which can be observed in response to heat stress. The entire heat stress stimulon of *B. subtilis* consists of three major modules, class I, II and III, of altogether five classes of specific heat shock genes (Völker et al., 1994; Hecker et al., 1996). Class I represents the HrcA-regulated specific heat stress regulon, while class II includes the general stress responsive genes regulated by the alternative sigma factor σ^B. The highly stress-relevant Clp-protease components ClpC, ClpE and ClpP controlled by the CtsR repressor protein are attached to class III. Analysis of mutant strains defective in genes of class I, class II or class III revealed that resistance to heat shock is not simply limited to the activation of one regulon and that malfunction of one module can be partly compensated by the others (Völker et al., 1992; Schulz et al., 1995; Völker et al., 1999).

The extensive investigation of the heat stress physiology in *B. subtilis* cells revealed a drastic reprogramming of gene expression after heat shock conditions. The heat stress stimulon of *B. subtilis* with its highly coordinated activation of different, i.e. specific and general, stress response modules may reflect in part the complexity of adaptive networks of bacterial cells in their natural habitats and can be regarded as a paradigm for an entire adaptive strategy.

The general stress response. When specific responses cannot eliminate threatening environmental conditions and stress or starvation periods extend, general defense strategies, which are activated prior or posterior, become more important in many bacteria. Besides sporulation, the general stress response of *B. subtilis* represents an alternative general strategy that does not include an obvious differentiation process of the cell (Price, 2002). The increased synthesis of general stress proteins is regarded to confer *B. subtilis* cells unspecific protection so that survival of extended periods of stress or starvation is enabled in form of vegetative cells (Hecker & Völker, 1998; Hecker & Völker, 2001).

Activation of the general stress response is controlled by the sigma factor σ^B, which was the first alternative sigma factor identified (Haldenwang & Losick, 1979; Igo et al., 1987). The *sigB* gene encoding σ^B is localized within an operon together with *rsbR*, *rsbS*, *rsbT*, *rsbU*, *rsbV*, *rsbW* and *rsbX* whose products contribute to the regulation of σ^B activity (Fig. 1A)(Kalman et al., 1990; Wise & Price, 1995). The entire operon is subject to

transcriptional initiation by the vegetative sigma factor σ^A. Therefore, a basic level of σ^B regulating proteins and σ^B itself is provided. The latter four genes of the operon are in addition subject to σ^B-dependent autoinduction at a σ^B promoter sequence (Kalman et al., 1990; Wise & Price, 1995). During exponential growth, silencing of the *sigB* operon is achieved due to the action of an antisigma factor RsbW that negatively controls basic levels of σ^B by building an inhibitory complex with σ^B (Benson & Haldenwang, 1993a; Benson & Haldenwang, 1993c). Upon imposition of stress or starvation, the antagonist protein RsbV is converted from its inactive into its active state by dephosphorylation and binds to RsbW (Dufour & Haldenwang, 1994; Völker et al., 1996). Consequently, the stable σ^B protein (Redfield & Price, 1996) is released from its inhibitory complex and the general stress regulon consisting of up to 150 genes is induced (Petersohn et al., 2001; Price et al., 2001). The accumulation of non-phosphorylated RsbV antagonist within this complex regulation of σ^B activation is attained by the action of two different signal-reception/transduction pathways (Völker et al., 1995a; Dufour et al., 1996).

After physical stress such as heat shock, salt and ethanol stress, the 2C-type protein phosphatase RsbU catalyses the dephosphorylation of RsbV~P, thereby activating the σ^B-dependent general stress response (Fig. 1B)(Völker et al., 1995a; Yang et al., 1996). RsbU itself is subject to a complex control: similar to RsbV, RsbW and σ^B, together with RsbT and RsbS, RsbU forms another 'partner-switching' module (Dufour et al., 1996; Kang et al., 1996; Yang et al., 1996). Under conditions of exponential growth, RsbT is occupied within a complex by RsbS. By the imposition of physical stress, the induced kinase activity of RsbT inactivates RsbS by phosphorylation. The RsbT protein in turn can now interact with RsbU, thereby inducing the RsbV-specific phosphatase activity of RsbU. The dephosphorylated form of RsbV is able to subsequently form a complex with RsbW. Thus, σ^B is set free and becomes active. The RsbS-specific kinase activity of RsbT is stimulated by physical stress. Regulation of RsbT kinase activity involves the interaction of RsbS which forms a supramolecular complex with RsbR able to capture RsbT (Akbar et al., 1997; Gaidenko et al., 1999; Chen et al., 2003). In addition, four recently identified RsbR homologues participate in regulation of RsbT (Akbar et al., 2001). Furthermore, the GTP-binding protein Obg was found to bind to RsbT and cofractionates RsbT, RsbS and RsbR with the ribosomes suggesting its participation in the signal perception of physical stress (Scott & Haldenwang, 1999; Scott et al., 2000). To date the detailed mechanism of signal perception is still unknown.
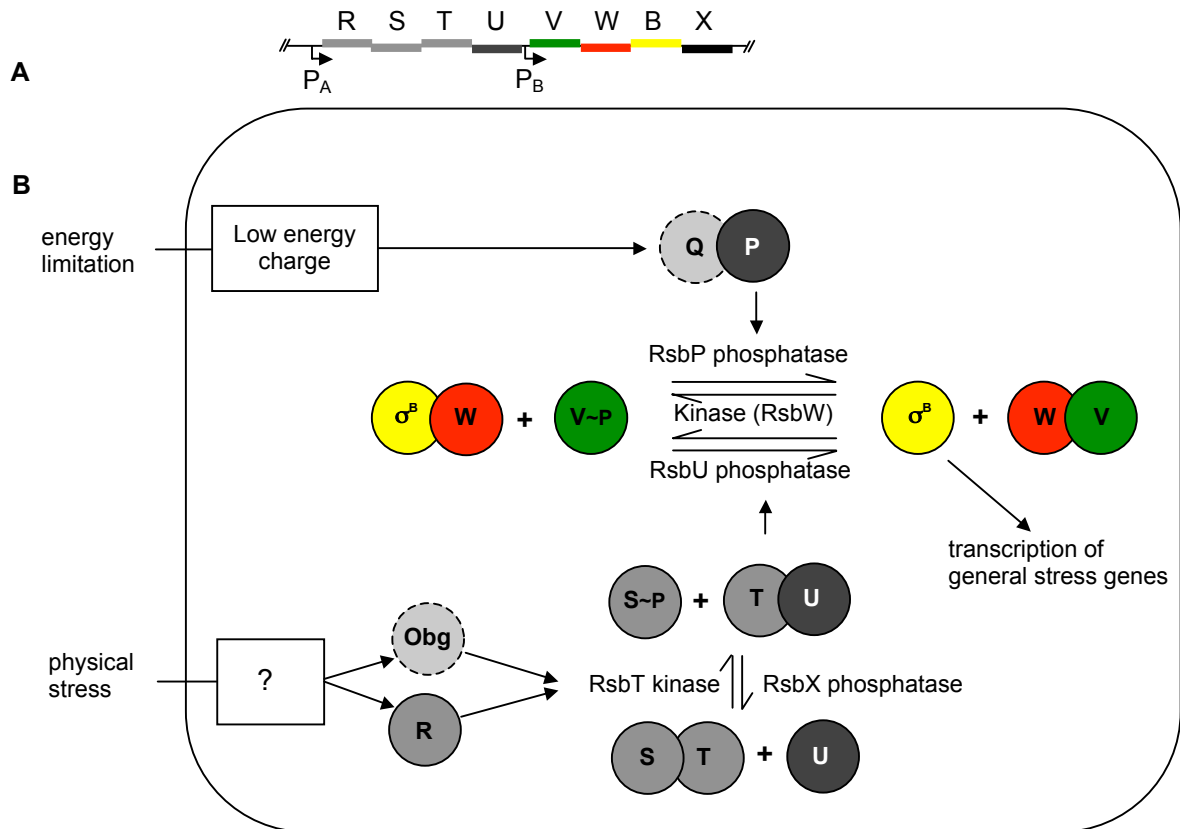
Fig. 1. Organization of the operon structure (**A**). Regulation of σ^B-activity (**B**). By the imposition of stress, antagonist protein RsbV~P is dephosphorylated by the action of a 2C-type phophatase, RsbU or RsbP. RsbV is now able to bind antisigma factor RsbW thereby preventing the complex formation of RsbW with σ^B. Upon release from its inhibitory complex, σ^B is active and effectively competes with vegetative σ^A for RNA polymerase binding. After energy depletion, RsbV-specific phosphatase activity of RsbP is stimulated, possibly by the action of RsbQ. After physical stress, a cascade of proteins triggers stimulation of RsbV-specific phosphatase activity of RsbU (for details, see text; modified from Hecker & Völker (2001)).

The general stress response displays only transient activity after exposure to physical stress. After 10 to 40 min, the σ^B-activity rapidly decreases by the action of the RsbX protein (Maul et al., 1995; Völker et al., 1995b). RsbX represents another phosphatase that dephosphorylates RsbS and thus counteracts the kinase activity of RsbT (Yang et al., 1996). The re-establishment of σ^B-silencing is realized due to the fact that RsbX amounts are increased by σ^B-dependent induction (Völker et al., 1997; Smirnova et al., 1998).

After energy depletion, the 2C-type protein phosphatase RsbP catalyzes the dephosphorylation of RsbV~P instead of RsbU (Fig 1B)(Völker et al., 1996; Vijay et al., 2000). In fact, the RsbU-dependent signaling pathway is not involved in triggering energy

depletion, and RsbP is dispensable at all after physical stress for activation of the general stress response. Since RsbP contains a N-terminal localized PAS-domain,[3] it is suggested that signal perception and regulation of phosphatase activity are both directly dependent on RsbP (Vijay et al., 2000). In addition to RsbP, the antisigma factor RsbW might also be involved in direct signal perception (Alper et al., 1994; Alper et al., 1996). RsbW displays a RsbV-specific kinase activity which is obviously to re-phosphorylate RsbV when conditions of stress or starvation ceases. Under conditions of low energy charge, a decrease of kinase activity of RsbW was observed *in vitro*, suggesting a potential activation of σ^B without the involvement of RsbP phosphatase (Alper et al., 1996). Recently, the participation of the α/β-hydrolase RsbQ was found to represent a positive regulator for RsbP phosphatase activity (Brody et al., 2001). Furthermore, the involvement of the ppGpp synthetase/stringent response regulatory protein RelA was reported in regulation of σ^B-activation after energy depletion (Zhang & Haldenwang, 2003).

The synthesis of general stress proteins is one of the earliest adjustments in *B. subtilis* cells in response to heat, salt, ethanol or acid stress or starvation of glucose, oxygen or phosphate (Völker et al., 1994; Hecker & Völker, 1998). More than 150 genes have been identified to be regulated by σ^B (Petersohn et al., 1999b; Petersohn et al., 1999c; Petersohn et al., 2001; Price et al., 2001). This huge number indicates a non-specific, protective function under threatening environmental conditions (Hecker & Völker, 2001). Although the information about σ^B-regulation and composition of the regulon increased, the physiological function of the general stress response remained hypothetical for a long time. Phenotypic studies of *sigB* mutant cells did not reveal any obvious participation in stress adaption or nutrient limitation. Furthermore, for a large number of genes no biochemical function could be identified. The heterogeneity of putative functions for other gene products with similarities to already identified proteins prohibited to draw conclusion on the entire function of the regulon. At an early stage, assumption was obtained that σ^B does not affect sporulation at all, since the loss of σ^B in a *sigB* mutant strain did not impaire the formation of endospores (Igo et al., 1987). First evidence for a role of the general stress response was deduced by the finding that glucose starved *sigB* mutant cells showed a decreased resistance in survival after hydrogen peroxide treatment compared to wild-type cells (Engelmann & Hecker, 1996). Likewise, resistance to cumene hydroperoxide was

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[3] PAS is an acronym designating protein signaling modules that monitor changes in light, redox potential, oxygen, small molecules, and overall energy level of a cell (**Taylor, B. L. & Zhulin, I. B. (1999).** PAS domains: internal sensors of oxygen, redox potential, and light. *Microbiol Mol Biol Rev* **63**, 479-506.).

observed to depend on the effect of general stress protein induction under conditions of glucose starvation and during exponential growth (Antelmann et al., 1996). Recently, extensive analysis revealed the comprehensive involvement of σ^B in cross-protection. Glucose-starved cells showed an increased resistance to freezing-thawing stress, UV-light and to a lesser extent also to salt and heat stress mediated by σ^B (Völker et al., 1999). Mutants in *sigB* were observed to be impaired in survival under more acidic or alkaline conditions in the stationary phase compared to the wild-type (Gaidenko & Price, 1998). Furthermore, wild-type cells showed an increased resistance after rifampin treatment under conditions of glucose starvation compared to *sigB* mutant cells (Bandow et al., 2002). Exposure to osmotic and temperature shifts was shown to induce cross-protection dependent on induction of the general stress response (Völker et al., 1999).

Oxidative stress

Oxygen. About 3.5 billion years ago, ancestral cyanobacteria started the oxygenation of the biosphere by photosynthetic lysis of water. The accumulation of molecular oxygen (O_2) led organisms to gain energy by utilization of O_2. Together with the accumulation of organic material, the efficient production of energy under aerobic conditions enabled the evolution of more complex organisms. To date, the atmosphere of the planet Earth consists of 78% nitrogen, 21% molecular oxygen and 1% other gases, here within 0.03% carbon dioxide (Elstner, 1990).

O_2 contains two spin-aligned, unpaired electrons in its molecular orbitals. Therefore, it cannot easily react with other molecules since typical molecules are equipped with pairs of antiparallel spinned electrons. Furthermore, the redox potential of O_2/O_2^- is relatively low with a value of –0.16 V, indicating that univalent electron-transfer reactions are possible but limited to electron donors with redox potentials more negative. Thus, O_2 is less reactive and therefore should be *per se* nontoxic for aerobic organisms. In biological systems, the spin restriction of O_2 can be abolished by the interaction with another paramagnetic center, e.g. with iron ions in proteins. O_2 can be utilized in various enzymic reactions. The most prominent benefit of O_2 for aerobically growing organisms is represented by the utilization of O_2 as terminal electron acceptor in the respiratory chain. However, upon the stepwise acceptance of electrons in univalent electron-transfer reactions, the reactivities of the resulting oxygen molecules are drastically increased (for further reading see Elstner, 1990).

Reactive oxygen species. Reactive oxygen species (ROS) derived from the reduction of dioxygen to superoxide (O_2^-), hydrogen peroxide (H_2O_2) or the hydroxyl radical ($OH^\bullet$) are highly reactive and can efficiently damage all biologically relevant molecules (Farr & Kogoma, 1991; Imlay, 2002).

The primary source of ROS generation in bacteria is suggested to be the metabolism of aerobically growing cells itself. In *E. coli*, several proteins have been identified that generate ROS when O_2 is available (McCord & Fridovich, 1969; Imlay, 1995; Messner & Imlay, 1999). All proteins known so far contain solvent-exposed flavin molecules that are able to transfer single electrons to acceptor molecules (Messner & Imlay, 1999). During their normal catalytic cycle, flavins accept or donate electrons from univalent redox cofactors, e.g. metal centers. To realize these transfer reactions, the flavosemiquinone form is stabilized, which in turn enhances the possibility of autoxidation reactions of the dihydroflavin residue with O_2, thereby producing O_2^- or H_2O_2 (Imlay, 2002). The degree of ROS generation is dependent on the availability of O_2 and the presence of flavin containing enzymes in the cell, whereby the adventitious reaction of different flavin-proteins with O_2 is widely disparate (Imlay, 2002). When the normal electron flow is blocked, the generation of ROS was observed to be markedly increased (Imlay, 2002). Instead of the primary O_2^- product, H_2O_2 can be released of the moiety when in prior a spin inversion of the flavosemiquinone or the approximate O_2^- molecule took place (Messner & Imlay, 1999).

O_2^- as well as H_2O_2 is more reactive than O_2. The O_2^- molecule is both a weak reducant and a moderate oxidant. Interestingly, it was demonstrated that O_2^- does not undergo reactions with amino acids, carbohydrate metabolites or nucleic acids, but O_2^- is known to react with particular iron sulfur clusters in proteins (Carlioz & Touati, 1986; Gardner & Fridovich, 1991; Brown et al., 1995). Since H_2O_2 behaves as an oxidant, several types of damage can be attributed to the reaction with H_2O_2 (Farr & Kogoma, 1991). But neither O_2^- nor H_2O_2 are suggested as strong enough as oxidizing agents to perform all forms of damage that had been identified so far. In fact, H_2O_2 reacts readily with reduced iron ions, thereby generating $OH^\bullet$ and OH^- in a reaction designated as Fenton reaction (Elstner, 1990). In aqueous environments, the highly destructive $OH^\bullet$ is the most powerful oxidant and oxidizes nearly all cellular compounds at diffusion-limited rates. Fe^{3+}, oxidized by H_2O_2, can be re-reduced by the action of O_2^- or other reducing agents and is able to participate as the transition metal in this reaction again (Haber-Weiss reaction).[4] In

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[4] The total formula of Haber-Weiss includes the metal-dependent conversion of H_2O_2 to $OH^\bullet$ and water.

general, the conversion of O_2^- to H_2O_2 respectively to $OH^\bullet$ is strongly dependent on the intracellular amount of O_2^- or H_2O_2 and transition metals, e.g. iron ions, and the availability of transition metals might have a strong influence on the level and type of oxidative damage.

Under conditions of aerobic growth, the accumulation of O_2^- and H_2O_2 inside bacterial cells is suggested to be minimized by the action of defense enzymes, e.g. by alkyl hydroperoxide reductase that is suggested to be the primary scavenger of H_2O_2 in growing *E. coli* cells and detoxifies H_2O_2 in addition to organic hydroperoxides (Seaver & Imlay, 2001). Moreover, bacterial cells are able to activate strategies in order to resist oxidative stress if the concentration of ROS increases (Farr & Kogoma, 1991). Under laboratory conditions, the stimulation of adaptive strategies is often achieved by the exogenous addition of oxidative agents. This mode of experimental operation resembles a naturally generation of H_2O_2 in the growth medium when H_2O_2 is formed by reaction of O_2 with substrates in aeriated cultures. In vivo, pathogenic bacteria encounter oxidative stress also from exogenous sources, if eukaryotic macrophages attack pathogens by respiratory bursts (Miller & Britigan, 1997). Besides the designated sources of ROS generation, increased levels of transition-metals, ionizing radiation and redox-active compounds may further enhance the intracellular concentration of ROS, whereby the latter two may occur at increased levels in natural habitats of the soil-living *B. subtilis* cells.

Oxidative damage. All biologically relevant macromolecules, i.e. proteins, nucleic acids and membrane lipids, are susceptible to oxidative damage. According to unequal characteristics in reactivity, generated ROS can damage target molecules with differing specificity resulting in various kinds of damage.

Oxidative modifications of proteins are assumed to impair or even inhibit a wide range of biochemical functions, resulting in the disruption of metabolic pathways (Berlett & Stadtman, 1997). In early studies, knowledge with respect to the mechanism of ROS damage to proteins was attained by exposing proteins to ionizing radiation. The effects of radiolysis in the presence of oxygen were found to differ significantly from effects observed under anaerobic conditions (Stadtman, 1993). Under aerobic conditions, considerable fragmentation of polypeptide chains was observed. In the absence of oxygen, formation of high-molecular-weight aggregates was reported. In general, the modification of polypeptide chains is initiated by $OH^\bullet$-mediated hydrogen abstraction at a α-carbon-atom with subsequent formation of a carbon centered radical. In the presence of oxygen, additional

oxidation reactions with O_2 and ROS yield transient imino derivatives and finally result in the fragmentation of the protein backbone. When availability of O_2 is limited, carbon centered radicals of amino acid residues in proteins tend to protein cross-linkages involving homologous or heterologous interactions between protein molecules (Stadtman, 1993; Berlett & Stadtman, 1997).

In addition to ROS mediated damage of the protein backbone, all amino acid residues of proteins were reported to be subjects to ROS attacks (Table 1). Aromatic amino acids and sulfur-containing amino acids are among the preferred targets of ROS. For example, cysteine and methionine residues are readily transformed into cysteine disulfides or cysteine sulfonic acids respectively methionine sulfoxides by nearly all forms of ROS (Berlett & Stadtman, 1997). Tyrosine residues are involved in intra- or interprotein tyr-tyr cross-linking reactions and aspartate, asparagine, and 2-oxo-imidazoline have been demonstrated to be the major products of histidine oxidation (Berlett & Stadtman, 1997). Another striking alteration is the introduction of carbonyl groups into proteins at lysine, arginine, proline or threonine amino acid residues by direct oxidation in a metal-catalyzed, site-specific reaction (Levine, 1983a; Stadtman, 1993). It is assumed that these metal-catalyzed reactions are 'caged' processes, i.e. amino acid residues at metal-binding sites are specific targets of ROS (Stadtman, 1993). The mechanism of carbonyl group formation involves the reaction of H_2O_2 with a Fe(II)-protein coordination complex yielding $OH^\bullet$, OH^- and Fe(III)-protein complexes. The $OH^\bullet$ subsequently mediates abstraction of a hydrogen atom in the amino acid residue side chain, resulting in the formation of an alkyl radical. Offloading the single electron followed by spontaneous hydrolysis finally results in the formation of an amino acid residue side chain located carbonyl group. Importantly, the

Amino acids	Oxidation products
Cysteine	Disulfides, cysteic acid
Methionine	Methionine sulfoxide, methionine sulfone
Tryptophan	2-, 4-, 5-, 6-, and 7-Hydroxytryptophan, nitrotryptophan, kynurenine, 3-hydroxykynurinine, formylkynurinine
Phenylalanine	2,3-Dihydroxyphenylalanine, 2-, 3-, and 4-hydroxyphenylalanine
Tyrosine	3,4-Dihydroxyphenylalanine, tyrosine-tyrosine cross-linkages, Tyr-O-Tyr, cross-linked nitrotyrosine
Histidine	2-Oxohistidine, asparagine, aspartic acid
Arginine	Glutamic semialdehyde
Lysine	α-Aminoadipic semialdehyde
Proline	2-Pyrrolidone, 4- and 5-hydroxyproline pyroglutamic acid, glutamic semialdehyde
Threonine	2-Amino-3-ketobutyric acid
Glutamyl	Oxalic acid, pyruvic acid

Table 1. Amino acids most susceptible to oxidation (from: Berlett & Sadtman, 1997).

reaction is assumed to represent a 'caged' process in which the highly reactive $OH^{\bullet}$ is not allowed to diffuse because of favored reaction with subjects in its closest surrounding. Carbonyl groups in amino acid side chains of proteins are readily detectable with 2,4-dinitrophenylhydrazine since they share the characteristics of α-ketoacyl derivatives (Levine et al., 1994; Shacter et al., 1994). The level of protein carbonyl formation has been utilized as an indicator for oxidative protein modification in tissue, cell and protein samples.

Oxidative damage has also been attributed to specific ROS. Typical characteristics of O_2^- damage involve the reaction of O_2^- with proteins containing exposed [4Fe-4S] clusters (Gardner & Fridovich, 1991; Brown et al., 1995). The attack of such clusters results in the release of iron and loss of activity. Furthermore, O_2^- is especially known to directly inhibit the synthesis of cysteine and aromatic amino acids in *E. coli* (Carlioz & Touati, 1986; Benov et al., 1996; Benov & Fridovich, 1999). H_2O_2 is known to preferentially mediate metal-catalyzed carbonyl groups in lysine, arginine, threonine and proline residues and is mainly responsible for the oxidation of cysteine residues and methionine to sulfoxide adducts.

Exposure of cells to various kinds of ROS or ROS-generating agents results in numerous types of DNA lesions. Primarily, oxidative damage of nucleic acid is linked to the deleterious effects of intracellular generated $OH^{\bullet}$ (Farr & Kogoma, 1991; Henle & Linn, 1997). Since both, the phosphodiester backbone of DNA and nitrogenous bases, provide ligands that are able to bind tightly to adventitious iron, DNA damage after peroxide exposure may be caused by metal-catalyzed local conversion of H_2O_2 to $OH^{\bullet}$ originated from H_2O_2 or O_2^- (Henle & Linn, 1997). Damage occurs at the deoxyribose components of DNA and may lead to strand breaks with 3'-phophate or 3'-phosphoglycolate termini. Strand breaks and structural lesions of nucleic acids are known to block replication and thus may contribute to lethality after oxidative stress. Interestingly, damage of proteins is not likely to be the cause of ROS mediated increase of cell death, but damage of DNA and membrane have been linked to killing by ROS (Farr & Kogoma, 1991). In contrast to strand lesions, base damage does not result in the pause of replication and is assumed to be not or only partly involved in the increase of cell death after ROS exposure. Attack on purine or pyrimidine bases results in the formation of e.g. 8-oxoguanine, hydroxymehtyl urea and urea (Marnett, 2000). Altogether, direct oxidation of bases leads to a numerous identified base alterations, including thymine glycols and DNA-protein cross-links, e.g. thymine-tyrosine. In addition to strand brakes, thymine glycol has been shown to block replication in vivo. The oxidative modification of bases is known to increase significantly the rate of

mutation events (Farr & Kogoma, 1991).

At least in mammalian systems peroxides trigger the peroxidation of membrane lipids. Since bacteria lack of polyunsaturated fatty acids that represent the primary target of ROS attacks in mammals, the appearance of membrane damage in bacteria might be limited to membrane protein damage (Imlay, 2002). In general, nearly all forms of ROS can initiate lipid peroxidation, thereby forming the highly reactive lipid peroxy radical. This reaction is perpetuated when more unsaturated fatty acids are involved and results in shortening of fatty acids. Consequently, the increase in membrane fluidity is followed by the loss of structural integrity (Farr & Kogoma, 1991). In addition, intermediates of lipid peroxide chain reactions have been shown to directly alkylate bases of DNA or to form cross-linkages with DNA (Marnett, 2000).

The forms of oxidative damage are innumerable and the listed types of damage are therefore fragmentary. Due to the continuous progress in the development of measurement technology one may expect a further increase in the list of identified target molecules and their resulting products after oxidative damage. The determination of damage patterns after challenging organisms with specific oxidative agents is rendered more difficult by the fact that a large number of secondary reactions including further oxidation reactions and reactions with organic molecules will take place during oxidative stress.

Prevention of oxidative damage. When levels of ROS are increased or antioxidant molecules are decreased, bacterial cells encounter oxidative stress. To prevent side effects of toxic oxygen species during respiration or to provide protection against increased levels of ROS, cellular defense and protection systems evolved. Aerobically growing organisms possess both constitutive and inducible defense strategies to minimize the oxidative threat. The existence of adaptive strategies to ROS were first discovered for *E. coli* and *Salmonella typhimurium* (Demple & Halbrook, 1983; Christman et al., 1985). Defense strategies of bacterial cells against oxidative stress include the action of scavenging enzymes, protection proteins and repair systems in order to maintain essentially physiological functions of the cellular systems and therefore secure the survival of a population or rather the entire species even under conditions of increased levels of ROS (for further reading see Farr & Kogoma, 1991; Pomposiello & Demple, 2002).

At least one form of superoxide dismutase (SOD) or SOD mimicking molecule is found in every aerobic bacterial cell (Fridovich, 1995; Fridovich, 1997). SOD's are able to

catalyze the dismutation of O_2^- to form H_2O_2 and molecular oxygen. Upon the metal ligand bound to the enzyme, three types of SOD's have been classified. Whereas manganese containing MnSOD's are found in prokaryotes and in eukaryotes as well, the existence of iron containing FeSOD's is limited to bacteria only (Fridovich, 1997). In contrast to earlier observations, the copper zinc containing CuZnSOD is also found in bacteria, but is localized in the periplasm (Fridovich, 1997). In the genome of *B. subtilis*, open reading frames for all three forms of SOD can be found (Kunst et al., 1997). For MnSOD (SodA), which is assumed to protect cells from oxidative stress during growth and sporulation, no function against oxidative stress could be identified so far (Casillas-Martinez & Setlow, 1997; Inaoka et al., 1999).

The elimination of H_2O_2 is carried out by catalases that redistribute electrons among H_2O_2 molecules, thereby generating oxygen and water (Switala & Loewen, 2002). Three catalases have been described in *B. subtilis*. An essential function in resistance to H_2O_2 has been elucidated for the vegetative catalase KatA (Loewen & Switala, 1987b). A second catalase KatE is under the sole control of σ^B, whereas the gene product KatX is regulated by σ^B and also by the sporulation sigma factor σ^F (Engelmann et al., 1995; Petersohn et al., 1999a). Unlike to the mode of operation of catalases, peroxidases catalyze the dismutation of H_2O_2 to H_2O by concomitant oxidation of NAD(P)H (Chae et al., 1994b). The NADPH-dependent alkyl hydroperoxide reductase system Ahp is suggested to perform the detoxification of organic hydroperoxides in *B. subtilis* (Antelmann et al., 1996; Bsat et al., 1996). However, in *E. coli* the alkyl hydroperoxide reductase is the primary scavenger of H_2O_2 that is generated at low levels during exponential growth (Seaver & Imlay, 2001). Both, catalases and peroxidases are haem-containing enzymes, which indicates the need for haem-biosynthesis proteins under conditions of catalase or peroxidase induction.

Several proteins contain conserved cysteine motifs that participate in electron transfer reactions and form transient disulfide bonds during their catalytic cycle, e.g. ribonucleotide reductase and phosphoadenosine phosphosulfate reductase of *E. coli* (Berardi et al., 1998; Lillig et al., 2003). To maintain the thiol-disulfide status under conditions of normal growth and especially after oxidative stress, bacteria possess members of the thioredoxin/glutaredoxin protein family (Aslund & Beckwith, 1999b). Since these proteins contain the cysteine residues organized in a 'C-XX-C' motif, they can readily react with proteins containing disulfide bonds, thereby restoring the thiol-status of the target protein (Aslund & Beckwith, 1999a). Thioredoxins and Glutaredoxins are themselves subject to

reduction, either by the direct action of a NADPH-dependent reductase, or due to transferring reductive power from the reductase by glutathione (Aslund & Beckwith, 1999b). In *B. subtilis*, only the thioredoxin system consisting of thioredoxin TrxA and thioredoxin reductase TrxB has been identified (Scharf et al., 1998). The presence of proteins homologous to TrxA encoded by open reading frames in the genome of *B. subtilis* was verified, but no indications for the participation in maintaining thiol status was reported so far (Kunst et al., 1997).

Since the integrity of DNA is crucial for the successful reproduction of organisms and nucleic acids are highly susceptible to oxidative damage, bacterial cells possess systems to defend and protect the bacterial chromosome (Henle & Linn, 1997; Marnett, 2000). Besides SOD and peroxidase activities that reduce the level of ROS, DNA protection proteins with iron sequestering activity may minimize the transition metal dependent conversion of H_2O_2 to $OH^{\cdot}$ in the vicinity of the bacterial chromosome. As well, the compact structure of the chromatin, either with histone-like proteins or PexB/Dps-homologues, may protect the bacterial DNA by this means under conditions of elevated levels of ROS. *B. subtilis* cells possess two homologous genes encoding PexB/Dps-like proteins. While *mrgA* is under peroxide specific control (Chen & Helmann, 1995), *dps* is subject to σ^A- and σ^B-dependent regulation (Antelmann et al., 1997a). In addition to the protection systems, a number of repair systems have been identified especially for the well investigated bacterium *E. coli* (for review see Henle & Linn, 1997; Marnett, 2000). By action of the base excision repair, damaged bases are recognized and excised by DNA glycosylases. The subsequent repair is accomplished in bacteria most likely by DNA polymerase I with preceding removal of base-less sugars or sugar fragments. Supplementary, mismatch repair systems and nucleotide excision repair as well as recombinational repair systems have been identified. In *B. subtilis*, challenge with DNA-damaging agents leads to the induction of about 20 genes classified to represent the SOS regulon controlled by a RecA/LexA dependent mechanism (Love et al., 1985). Besides the UvrABC endonuclease which catalyzes the excision repair, most functions of the SOS gene products are still unknown.

Oxidative stress in *B. subtilis*. Since *B. subtilis* is thought to gain energy under aerobic conditions primarily by transferring electrons from carbon sources to oxygen, it had been suggested that the generation of ROS plays an important role also in this species. The identification of adaptive strategies in other respiring bacteria, especially in *E. coli* and

S. typhimurium (Demple & Halbrook, 1983; Christman et al., 1985), underlined the suggestion that an inducible response to eventually occurring ROS should also exist in *B. subtilis* cells. Evidence for an inducible response was first obtained by the observation that pretreatment with low peroxide concentrations led to a nearly complete resistance to otherwise lethal doses of H_2O_2 (Murphy et al., 1987). In addition, inducible catalase activity was reported and several catalase enzymes could be identified (Loewen & Switala, 1987a; Loewen & Switala, 1987b; Loewen & Switala, 1987c; Bol & Yasbin, 1991). It was shown that cells without a functional vegetative catalase were significantly more sensitive to H_2O_2 than wild-type cells (Bol & Yasbin, 1990). With respect to the mutagenicity of ROS, the induction of DNA-damage-inducible loci after peroxide challenge was tested. It turned out that this SOS-like response was indeed activated due to H_2O_2 treatment and is dependent on a functional *recA* (formerly *recE*) allel (Bol & Yasbin, 1990). In 1995, Chen and coworkers reported the identification of MrgA as a member of the Dps/PexB family of DNA binding proteins (Chen & Helmann, 1995). The search for mutant strains, displaying derepression of the *mrgA* gene, led the authors to propose the existence of a peroxide specific repressor protein in *B. subtilis* whose activity is regulated by peroxide and metal-ions (Chen et al., 1995). Upon the availability of *B. subtilis* genome sequence, within the group of three Fur-homologue proteins, the peroxide repressor protein, designated PerR, was finally identified (Bsat et al., 1998). After oxidative stress, PerR is suggested to be inactivated by H_2O_2 at a metal center of the protein, thereby loosing its DNA binding ability to the otherwise specifically recognized motif, i.e. the per box, in front of the regulated genes (Bsat et al., 1996; Herbig & Helmann, 2001). The derepression of the PerR regulon leads to increased synthesis of the vegetative catalase KatA, the alkyl hydroperoxide reductase AhpC/AhpF, the DNA protecting protein MrgA and the heme biosynthesis proteins HemA, HemX, HemC, HemD, HemB, and HemL (Bsat et al., 1996). In addition, the genes, which encode the iron uptake regulator Fur, the zinc uptake system ZosA (formerly YkvW) and PerR itself, are members of the PerR regulon (Herbig & Helmann, 2001; Helmann et al., 2003). By means of two-dimensional gel electrophoresis, first attempts were made to analyse the response of *B. subtilis* to the exogenous addition of O_2^-. Treatment with paraquat, which causes O_2^- stress, displayed an enhanced synthesis of a set of specific proteins in addition to proteins synthesized after H_2O_2 challenge (Antelmann et al., 1997b; Bernhardt et al., 1999). No O_2^- specific regulator was identified in *B. subtilis* so far. Survival analysis throughout the growth phase revealed that stationary phase *B. subtilis* cells are more resistant to H_2O_2 than exponentially growing cells (Dowds

et al., 1987). It was demonstrated by Engelmann & Hecker (1996) that this resistance is at least partly dependent on σ^B. Furthermore, the general stress response provides resistance to the radical generating cumene hydroperoxide in stationary phase and exponentially growing cells (Antelmann et al., 1996). Detailed investigations of the σ^B-regulon have revealed the possible involvement of proteins homologous to detoxifying catalase and DNA protecting proteins induced at the onset of stationary phase in *E. coli* (Von Ossowski et al., 1991; Almirón et al., 1992; Engelmann et al., 1995; Antelmann et al., 1997a). While the DNA binding protein Dps was found to be essential for the survival of glucose-starved *B. subtilis* cells treated with H_2O_2, the σ^B-dependent katalase KatE does not seem to be involved in this resistance (Engelmann & Hecker, 1996; Antelmann et al., 1997b).

Scope of this thesis

In the present work, aspects of gene expression pattern, protein damage and participation of general stress proteins under conditions of oxidative stress were analyzed.

B. subtilis responds to increased levels of ROS with the activation of defense and protection systems. At the beginning of this work, the knowledge about the change of gene expression pattern in response to oxidative stress was limited to results obtained by two-dimensional gel electrophoresis as the sole global identification strategy. To complete the description of gene expression pattern, the mRNA and protein synthesis were analyzed by means of DNA macroarray technique in combination with two-dimensional gel electrophoresis respectively.

The induced protection of starved *B. subtilis* cells against H_2O_2 was reported to involve essentially the σ^B-dependent general stress response. To identify the role of σ^B with respect to the prevention of protein damage under conditions of oxidative stress, protein carbonyl amounts were analyzed after derivatization with 2,4-dinitrophenylhydrazine. The corresponding hydrazone was determined by immunoanalysis.

The availability of strains with mutations in σ^B-dependent genes enabled the search for σ^B-dependent proteins that are involved in providing resistance to multiple stresses. Within this work, the screening for proteins involved in resistance to oxidative stress was carried out after reliable assay conditions were established.

The physiological function of thioredoxin in *B. subtilis* was not investigated up till now. In order to provide first indications about thioredoxin function, a conditional mutant strain was analysed by means of growth monitoring and proteomics.

Transcriptome and Proteome Analysis of *Bacillus subtilis* Gene Expression in Response to Superoxide and Peroxide Stress

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Summary. The Gram-positive soil bacterium *Bacillus subtilis* responds to oxidative stress by the activation of different cellular defense mechanisms. These are composed of scavenging enzymes as well as protection and repair systems organized in highly sophisticated networks. In this study, the peroxide and the superoxide stress stimulons of *B. subtilis* were characterized by means of transcriptomics and proteomics. The results demonstrate that oxidative stress responsive genes can be classified into two groups. One group encompasses genes which show similar expression patterns in the presence of both reactive oxygen species. Examples are the members of the PerR and the Fur regulon which were induced by peroxide and superoxide stress. Similarly, both kinds of stress stimulated the activation of the stringent response. The second group is composed of genes primarily responding to one stimulus, like the members of the SOS regulon which were particularly upregulated in the presence of peroxide and, many genes involved in sulfate assimilation and methionine biosynthesis which were only induced by superoxide. Several genes encoding proteins of unknown function could be assigned to one of these groups.

Introduction

The oxygenation of the biosphere which was started by the ancestors of cyanobacteria forced the evolution of aerobic respiration as well as of systems preventing damage by toxic oxygen species. Whereas dioxygen itself is less reactive and therefore nontoxic, reactive oxygen species (ROS) derived from the reduction of dioxygen to superoxide (O_2^-), hydrogen peroxide (H_2O_2) or the hydroxyl radical ($OH^\bullet$) are highly reactive and able to efficiently damage nucleic acids, membrane lipids and proteins (Farr & Kogoma, 1991; Imlay, 2002).

The O_2^- anion is known to attack enzymes with exposed [4Fe-4S] clusters, which results in the release of iron and loss of activity (Gardner & Fridovich, 1991;

Brown et al., 1995). Furthermore, O_2^- directly inhibits the synthesis of cysteine and aromatic amino acids in *Escherichia coli* (Carlioz & Touati, 1986; Benov et al., 1996; Benov & Fridovich, 1999). By spontanous dismutation or in the course of its enzymic detoxification, O_2^- is rapidly converted to H_2O_2.

Peroxides represent weak oxidizing agents known to react with cysteinyl-thiols in proteins by formation of disulfide bonds or sulfonic acid derivatives (Imlay, 2002). H_2O_2 is able to generate carbonyl groups in lysine, arginine, threonine and proline residues and to oxidize methionine to sulfoxide adducts (Stadtman, 1993). Peroxidation of membrane lipids is triggered by peroxides at least in mammalian systems (Imlay, 2002). Most significantly, H_2O_2 reacts with reduced iron ions to form $OH^•$, which in turn oxidizes most cellular compounds at diffusion-limited rates (Farr & Kogoma, 1991; Henle & Linn, 1997). Since the phosphodiester backbone and nitrogenous bases both provide ligands that can tightly bind adventitious iron, DNA damage after peroxide exposure may be caused by metal-catalyzed local conversion of H_2O_2 to $OH^•$ (Henle & Linn, 1997).

In aerobically growing cells, significant amounts of O_2^- and H_2O_2 are generated by enzymic misdirection of electrons to dioxygen. It is assumed that flavin-dependent transfer reactions of the respiratory chain are primarily responsible for the generation of ROS (Imlay & Fridovich, 1991; Messner & Imlay, 1999). In addition to the autoxidation of cellular components, exposure to ionizing radiation, redox-active compounds or transition-metals and depletion of antioxidants may contribute to the intracellular formation of ROS. If the amount of ROS increases to toxic levels, cells encounter oxidative stress.

In bacteria, oxidative stress is sensed by specific transcriptional regulators able to activate defense mechanisms when the ROS concentration exceeds a critical level (Farr & Kogoma, 1991; Storz & Zheng, 2000; Pomposiello & Demple, 2002). In *E. coli*, peroxides are monitored by the response regulator OxyR (Christman et al., 1989). In the presence of peroxides, an intramolecular thiol-disulfide switch of cysteine residues causes activation of OxyR, which subsequently leads to the expression of peroxide defense proteins including the H_2O_2 detoxificating catalase HPI (VanBogelen et al., 1987; Zheng et al., 1998). After O_2^- treatment, the oxidation of a $[2Fe-2S]^+$ cluster of the SoxR regulatory protein to $[2Fe-2S]^{+2}$ activates the SoxRS system and induces the O_2^- specific response (Gaudu et al., 1997; Hidalgo et al., 1997). Among other targets of SoxRS, a Mn-dependent superoxide dismutase catalyzing the dismutation of O_2^- to H_2O_2 is

synthesized at elevated levels (Walkup & Kogoma, 1989; Greenberg et al., 1990; Pomposiello & Demple, 2002).

In the soil-bacterium *Bacillus subtilis,* protection against inorganic peroxides is primarily mediated by the induction of specific stress proteins which are controlled by the repressor protein PerR (Chen et al., 1995; Bsat et al., 1998). In contrast to OxyR of *E. coli,* the Fur-homologous PerR regulator requires a metal cofactor for DNA binding (Herbig & Helmann, 2001). H_2O_2 is suggested to react at a metal centre of the protein, thereby impairing the DNA binding ability (Herbig & Helmann, 2001), which leads to increased synthesis of the vegetative catalase KatA, the alkyl hydroperoxide reductase AhpC/AhpF, the DNA protecting protein MrgA and the heme biosynthesis proteins HemA, HemX, HemC, HemD, HemB, and HemL (Bsat et al., 1996). In addition, the genes encoding the iron-uptake regulator Fur, the zinc-uptake system ZosA (formerly YkvW) and PerR itself are members of the PerR regulon (Herbig & Helmann, 2002; Helmann et al., 2003). The observed induction of genes encoding peroxide stress proteins after challenging the cells with low levels of H_2O_2 causes a nearly complete resistance of *B. subtilis* to otherwise lethal concentrations of H_2O_2 (Dowds et al., 1987; Murphy et al., 1987). In contrast to the PerR-dependent H_2O_2 protection, resistance to organic peroxides is mediated by the OhrR repressor (Fuangthong et al., 2001). Up to now, no O_2^--specific regulator was found in *B. subtilis*. Previous studies using 2D-PAGE revealed that addition of paraquat, which causes O_2^- stress, triggers the enhanced synthesis of a set of specific proteins which are not induced by H_2O_2 (Antelmann et al., 1997b; Bernhardt et al., 1999).

In this study, we analyzed the global gene expression of *B. subtilis* in response to oxidative stress. Variations in gene expression after treatment with H_2O_2 or the O_2^--generating agent paraquat were monitored at the level of transcription using DNA macroarray hybridization and at the level of protein synthesis by two-dimensional gel electrophoresis. The results allowed classification of oxidative-stress-responsive genes into two groups: those that exhibited similar expression patterns in the presence of both ROS, and those that responded more strongly to one stimulus or the other. Many genes encoding proteins of so far unknown functions could be assigned to one of these groups, suggesting functions in the context of coping with oxidative stress conditions. The present study represents a first step towards the functional characterization of these genes.

Methods

Proteome analysis. The *B. subtilis* wild-type strain 168 (Anagnostopoulos & Spizizen, 1961) was cultivated aerobically at 37°C in a synthetic medium (Stülke et al., 1993). Conditions of oxidative stress were achieved by the addition of H_2O_2 to a final concentration of 58 µM or paraquat to a final concentration of 100 µM to exponentially growing cells at an OD_{500} of 0.4. Radioactive labelling was performed for 5 min with 10 µCi/mL of L–[^{35}S]methionine (Amersham Pharmacia Biotech). Cells were harvested before and 10 min after addition of H_2O_2 or paraquat. Preparation of protein extracts and 2D gel protein gel electrophoresis were carried out as described previously (Büttner et al., 2001). Crude protein extracts containing 60 µg protein for analytical gels and 250 µg protein for preparative gels were loaded onto IPG-strips in the pH range 4-7 (Amersham Pharmacia Biotech) for the first dimension of 2D electrophoresis. Preparative gels were stained with Sypro Ruby (Molecular Probes) according to the recommendations of the manufacturer. Autoradiographs of analytical gels were collected with the PhosphorImager SI Scanner (Molecular Dynamics), and data were analyzed by comparison of the 2D protein patterns of control and stressed cultures using Delta2D software (Version 3.1; Decodon). Identification of proteins by means of MALDI-TOF-MS was performed as previously described (Eymann et al., 2002).

Transcriptome analysis. Cultivation of *B. subtilis* 168 and induction of oxidative stress was performed as described for the proteome analysis. Cell harvesting, preparation of RNA, and macroarray analysis with Panorama *B. subtilis* gene arrays and specific cDNA labeling primers (Sigma-Genosys) were performed as described by Eymann *et al.* (2002). The total RNA was checked by Northern blot analysis. For each condition, four macroarray experiments were carried out using two independently isolated RNA preparations and two different array batches. Quantification of the hybridization signals using the ArrayVision software (version 5.1, Imaging Research) was performed as described by Eymann et al. (2002). Gene-specific expression level ratios were calculated by dividing the mean of the normalized, artifact-reduced volumes of the parallel replica spots on the stress array (H_2O_2 or paraquat) by the corresponding values of the control array for upregulated expression or reciprocally for downregulated expression. Expression level ratios ≥ 3 in at least three of four experiments per condition were considered as significant. Final evaluation of the macroarray data included the consideration

of putative operons derived from the genome sequence using the SubtiList data-base (http://genolist.pasteur.fr/SubtiList/) as well as previously known transcriptional units.

In order to facilitate presentation of the expression data, arithmetic mean and standard deviation calculated from all four expression level ratios are given for every significantly upregulated gene in Table 1. The datasets for all genes significantly up- and downregulated by peroxide and paraquat stress including all expression level ratios are available at http://mic.sgmjournals.org. The complete dataset of the transcriptome analysis can be obtained upon request.

Northern analysis. The Northern hybridization procedure was carried out as described previously (Eymann *et al*., 2002). Chemiluminescence was detected with the Lumi-Imager (Roche Diagnostics) and transcript sizes were determined by comparison with an RNA size marker (Invitrogen). The marker band positions are depicted on the left in all Northern blot images. The DIG-labeled RNA probes were synthesized by *in vitro* transcription with T7 RNA polymerase and specific PCR products as templates. Synthesis of the templates by PCR was performed using the following oligonucleotide pairs: for the *dhbF* probe, DHBF-5' (5'-ATGCCTGATACAAA AGATCT-3') and DHBF-3' (5'-CTAATACGA CTCACTATAGGGAGACAGCCAGAATTG ACGATCCT-3'); for *fhuD*, FHUD-5'-(5'-ATG ACCCATATATACAAGAA-3') and FHUD-3' (5'-CTAATACGACTCACTATAGGGAGATT TTCCGTTCGTTTGCATAA-3'); for *ykuP*, YKUP-5'(5'-TTGGCGAAGATTTTGCTCGT-3') and YKUP-3' (5'-CTAATACGAC TCACTATAGGGAGACCTCATTACTGTAT CAAAGG-3'); for *yxeB*, YXEB-5' (5'-ATG AAAAAGAACATATTGCT-3') and YXEB-3' (5'-CTAATACGACTCACTATAGGGAGACC AGAATTCGCCCTTGTCTG). The underlined sequences indicate the T7 promoter region.

Results and discussion

Oxidative stress, growth behavior and global gene expression profiling. In order to obtain a comprehensive view of gene expression and protein synthesis patterns in response to oxidative stress, transcriptome analyses using DNA macroarrays and proteome analyses using 2D protein gel electrophoresis were performed. To induce peroxide- or superoxide-specific stress responses in exponentially growing cells, concentrations of 58 µM H_2O_2 or 100 µM paraquat were used. The influence of the two stimuli on the growth behavior was different: cells challenged with H_2O_2 continued growth, albeit with a reduced rate, whereas addition of paraquat caused cessation of cell growth

(data not shown). The RNA preparations used in the transcriptome analyses were first checked for quality by RNA electrophoresis and subsequent Northern hybridizations using *katA*- and *trxA*-specific probes. As expected, both genes showed a strong transcriptional induction by both stimuli (data not shown).

Characterization of transcriptome and proteome after exposure to H₂O₂ and paraquat. The transcriptome analysis revealed 92 genes exhibiting significantly elevated and 97 genes exhibiting significantly reduced mRNA levels after H_2O_2 challenge. After exposure to paraquat, the expression of 129 genes was found to be induced at the level of mRNA while the expression of 169 genes was decreased. From these genes, 51 were up- and 87 were downregulated by peroxide as well as superoxide stress. The accompanying proteome analysis demonstrated enhanced synthesis for about 55 proteins after peroxide challenge and decreased synthesis for about 150 proteins (Fig. 1A). About 65 proteins were found to be synthesized at higher rates and 200 at lower rates after exposure to paraquat (Fig. 1B). Around 20 proteins were present in higher and 140 proteins in lower amounts after peroxide as well as after paraquat treatment. The expression data for upregulated genes are summarized in

Table 1; results for the various classes of genes are discussed below.

PerR regulated genes. Previous analyses of the peroxide stimulon identified seven transcriptional units as direct targets of PerR (Bsat et al., 1996; Helmann et al., 2003). In our analysis, nearly all of these known members of the PerR regulon were found at significantly elevated expression levels after H_2O_2 and paraquat challenge. The highest induction factors of all in this study were detected for the PerR-regulated genes *katA* (145.8, 171.2) and *mrgA* (85.5, 163.6) encoding the vegetative catalase KatA and the DNA-protection protein MrgA. Significant induction after both stimuli was also detected for the transcriptional units *ahpCF*, *zosA* and *fur* which encode the alkylhydroperoxidase AhpCF, the zinc-uptake protein ZosA and the iron-uptake regulatory protein Fur, respectively. The haem biosynthesis operon *hemAXCDBL* exhibited only a weak upregulation by H_2O_2 and no significant induction after O_2^- stress. The *perR* gene encoding the PerR repressor protein was found to be induced after paraquat challenge but did not reach the significance threshhold of a threefold increase after peroxide stress. In addition to the known PerR targets, our analysis identified three putative transcriptional units (*yoeB*, *yuiAB*, *yxbC*) exhibiting elevated expression levels solely after paraquat

Fig. 1. Protein synthesis patterns of *B. subtilis* 168 under conditions of peroxide stress (**A**) and O_2^- stress (**B**). Protein extracts were prepared from cells growing exponentially in defined medium before and 20 min after addition of H_2O_2 (final concentration 58 µM) or paraquat (final concentration 100 µM). Radioactive labeling was performed for 5 min with 10 µCi/mL of L-[^{35}S]methionine. Proteins were separated as described in Methods. Dual-channel images were generated by combining the obtained autoradiograms. Protein spots that decreased in intensity under stress conditions are colored green and spots that increased are colored red.
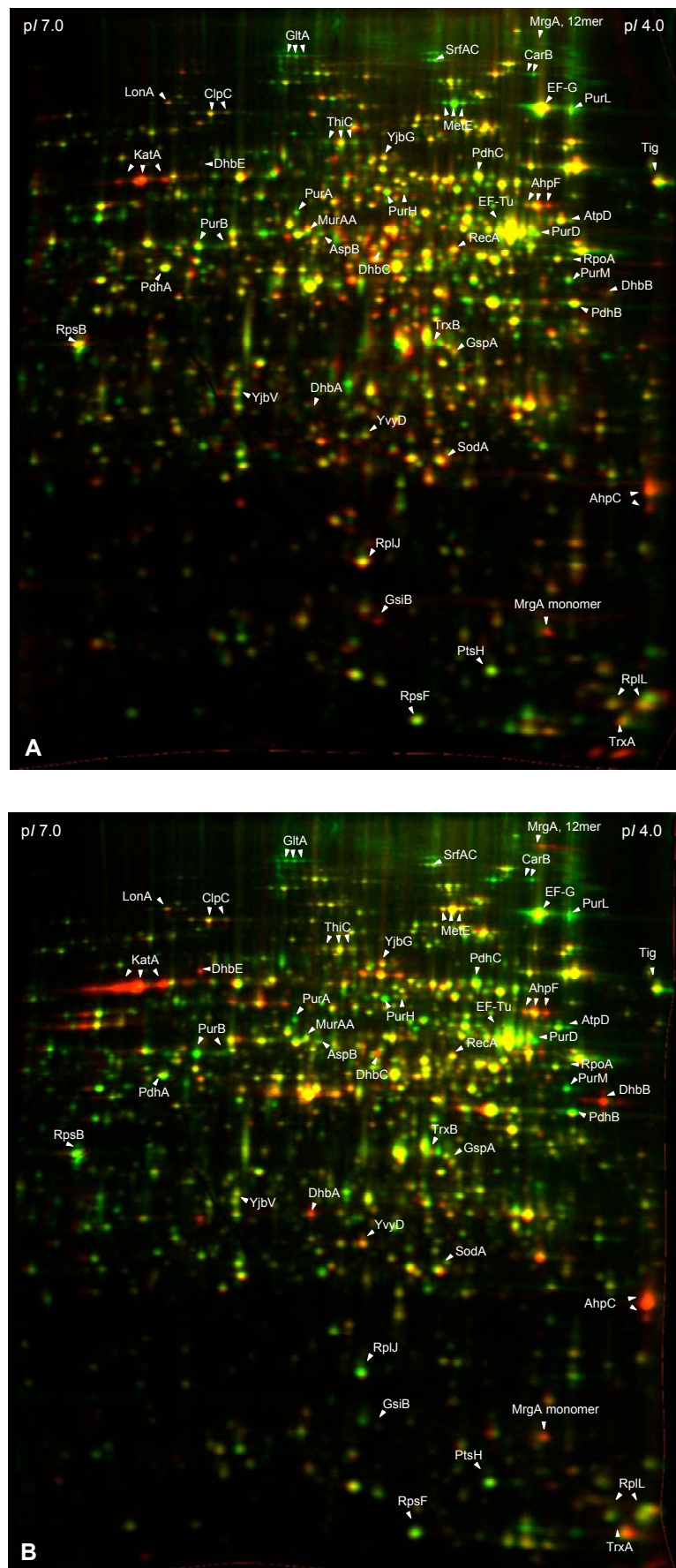
challenge. These genes were previously shown to be upregulated in a *perR* mutant by Helmann *et al.* (2003), indicating direct or indirect regulation *via* PerR. Apart from the *hem* operon and the *fur* gene, the detected transcriptional units were more strongly induced by O_2^- than by H_2O_2.

The primary defense against the effects of inorganic peroxides involves the induction of the PerR-dependent stress response. The activation of this peroxide-specific response after exposure to O_2^- observed in our analysis may reflect the dismutation of O_2^- to H_2O_2 by either spontaneous dismutation or as part of antioxidant strategies. Alternatively, direct inactivation of PerR by O_2^- can not be excluded. In *E. coli*, the gene expression patterns after O_2^- and H_2O_2 treatment also show a partial overlap, including all peroxide-specific stress proteins belonging to the OxyR regulon (Greenberg & Demple, 1989). The overlap of the O_2^- and peroxide responses in *B. subtilis* including members of the PerR regulon has already been demonstrated by means of proteomics (Antelmann et al., 1997b; Bernhardt et al., 1999).

Fur-Regulon. The *fur* gene encoding the ferric uptake regulator was found to be induced after oxidative stress, most probably due to PerR-dependent derepression, which was previously demonstrated (Fuangthong et al., 2002). The *fur* induction could indicate a shutdown of the cellular iron uptake to prevent further generation of $OH^•$, since intracellular iron promotes formation of $OH^•$ from H_2O_2 via Fenton chemistry in the presence of ROS. However, the Fur repressor protein itself appeared to be inactive under the conditions tested. In our study, 11 of the 25 probable transcriptional units described to be upregulated in a *fur* knockout mutant (Baichoo et al., 2002) exhibited increased mRNA levels after peroxide and O_2^- stress, indicating derepression of the Fur regulon. In *E. coli*, *fur* was also reported to show increased expression after H_2O_2 and O_2^- stress which is mediated by OxyR and SoxRS (Zheng et al., 1999). The observation of high Fur protein amounts after oxidative stress prompted these authors to speculate that iron uptake regulation might not be the only function of Fur. It was proposed that the Fur protein could sequester iron in order to deprive DNA of metal ions or to catalyze the breakdown of H_2O_2 directly (Zheng et al., 1999). This could also be the case for the *B. subtilis* Fur protein.

On the other hand, the induction of genes with functions in iron uptake which was detected in our analysis could represent an adaptive response to iron limitation. The activity of many defense proteins, e.g. catalases, requires metal ion
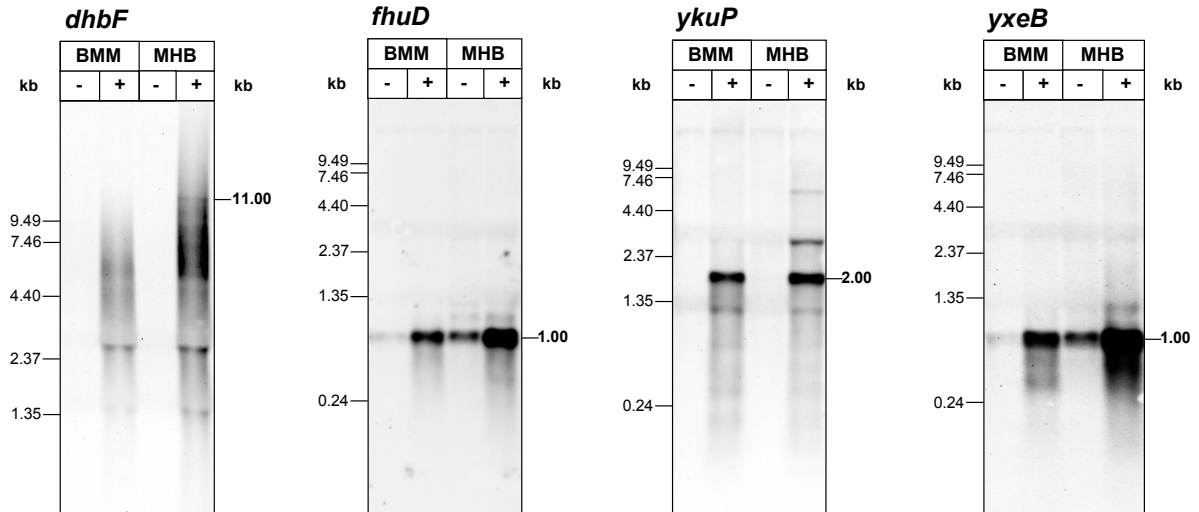
Fig. 2. Northern analysis of *dhbF*, *fhuD*, *ykuP* and *yxeB* in synthetic medium (Belitsky Minimal Medium, BMM) and rich medium (Mueller-Hinton Broth, MHB). RNA was prepared from cells growing exponentially before (-) and 10 minutes after (+) addition of H_2O_2 to a final concentration of 58 µM. Electrophoretic separation of the RNA (5 µg per lane) was performed using 1.2 % gels (*fhuD*, *ykuP*, *yxeB*) or 0.6 % gels (*dhbF*).

incorporation. In an approach similar to our study, Helmann et al. (2003) did not detect induction of the Fur regulon using a comparable H_2O_2 concentration. Since the experiments reported by these authors were performed in rich medium in contrast to the synthetic medium used in our experiments, we speculated that the different expression patterns might have resulted from differing metal concentrations in these media. To verify this hypothesis, cells were grown in the synthetic medium used in our transcriptome analysis (Belitsky Minimal Medium, BMM) as well as in the rich medium used by Helmann and coworkers (Mueller-Hinton Broth, MHB) and RNA was prepared before and after addition of H_2O_2. These RNA preparations were used in Northern hybridizations with probes for mRNA specified by *dhbF*, *fhuD*, *ykuP* and *yxeB*. These genes were previously demonstrated to represent direct targets of Fur (Baichoo *et al.*, 2002) and were significantly upregulated after addition of H_2O_2 in our transcriptome study. The results of these Northern experiments are shown in Fig. 2.

In both media, all four genes were strongly induced at the mRNA level by peroxide stress. According to the *B. subtilis* genome sequence (Kunst *et al.*, 1997), the *dhbF* gene can be predicted to be transcribed as part of a 11.0 kb *dhbACEBF* mRNA. This large transcript was detected in rich medium after addition of H_2O_2. Although the distinct 11.0-kb band was not detected in minimal medium, the strong smeary signals clearly indicated significant induction. The *fhuD*- and the *yxeB*-specific probes hybridized to monocistronic mRNAs of 1.0 kb in length, whereas the most abundant mRNA detected using the *ykuP*

probe indicates a tricistronic 2.0 kb *ykuNOP* transcript. The levels of *fhuD*- and *yxeB*-specific mRNA before and after addition of H_2O_2 were higher in rich medium compared to synthetic medium. This may also apply to *dhbF* and *ykuP*, as the induced mRNA levels of these genes were also clearly higher in rich medium. However, these transcripts were not at all detectable in the absence of H_2O_2, which precludes statements concerning the basic mRNA levels.

The results of the Northern hybridizations unambiguously demonstrated induction of the Fur regulon by H_2O_2 in synthetic as well as in rich medium. Therefore, it can be excluded that different metal concentrations in the media were responsible for the differences between our results and those obtained by Helmann et al. (2003). It has to be supposed that the use of different techniques for transcriptome analysis (macroarrays versus microarrays) accounts for the observed differences, maybe as a result of differential sensitivities.

σ^B-Regulon. Although oxidative stress does not represent a typical inducer of the σ^B-dependent general stress response, 12 probable transcriptional units described to be regulated by σ^B (Petersohn et al., 2001) were found to have significantly elevated expression levels after peroxide treatment,

among them the *csbD*-, *gspA*- and *yjgD*-specific mRNAs. In addition, several of the σ^B-dependent genes were also induced by paraquat treatment. Inspection of the induction factors of the other known σ^B-dependent genes revealed that the majority were slightly induced after peroxide treatment even though the ratios did not match the criterion of significance. This observation is in accordance with the findings of Helmann et al. (2003), who also reported induction of σ^B-dependent genes after H_2O_2 treatment. Further studies will reveal if the activation of the σ^B regulon is accomplished by limitation of energy via the RsbP regulatory protein or if peroxide stress activates the general stress response by physical stress via the RsbU-dependent signaling cascade (Hecker & Völker, 2001).

CtsR-Regulon. The expression of the CtsR-regulated operon *ctsR-mcsA-mcsB-clpC*, which belongs to the class III of heat shock genes (Krüger & Hecker, 1998), was increased by both oxidative stimuli. The two genes, *radA* and *yacK*, which are located immediately downstream of the *clpC* operon also exhibited oxidative stress induction, indicating transcriptional readthrough of the terminator structure downstream of *clpC* under these conditions. The other members of the class III of heat shock genes, the monocistronic

transcribed genes *clpE* and *clpP*, showed around twofold increased mRNA amounts, thereby missing the criterion of significance, under one or both conditions. It was previously shown that class III heat shock genes controlled by CtsR are strongly induced by the disulfide stress generating agent diamide (Leichert et al., 2003). Disulfide stress may be considered as a subgroup of oxidative stress since peroxides are assumed to act as disulfide generating agents and could represent the link between oxidative and heat stress.

Sulfur-limitation-regulated genes.
Several genes encoding proteins involved in sulfur assimilation and synthesis of the sulfur-containing amino acids cysteine and methionine were induced after exposure to paraquat. In contrast, no change or even a downregulation was found for most of these genes after H_2O_2 challenge. Significant induction after exposure to O_2^- was observed for 6 of the 11 transcriptional units described to form the S box regulon. As the S box regulatory mechanism was recently reported to be based on a direct interaction of available S-adenosyl-methionine with the mRNA leader regions, the induction of this regulon indicated methionine limitation (Grundy & Henkin, 1998; Mandal et al., 2003; McDaniel et al., 2003). The genes *yxjG* and *yxjH,* which are also predicted to represent members of the

S box regulon, exhibited an induction around twofold, thereby missing the criterion of significance.

Elevated expression was also found for the *ssuABCDygaN* operon, which encodes proteins for uptake and utilization of aliphatic sulfonates in the context of sulfur assimilation (van der Ploeg et al., 1998). This operon is not regulated by the S box mechanism and is repressed by sulfate and cysteine, which also holds true for the two recently characterized operons *ytmIJKLMNO-ytnI-ribR-ytnLM* and *yxeKLMNOPQ*, encoding proteins predicted to be involved in the utilization of alternative sulfur sources (Coppee et al., 2001; Auger et al., 2002).

Altogether, the data strongly suggest a O_2^- stress induced sulfur limitation in *B. subtilis*. As the iron-sulphur cluster containing sulfite reductase is not inactivated by O_2^- in *E. coli*, this phenotype may not result from a specific damage of this enzyme (Messner & Imlay, 1999). The reduction of paraquat within its O_2^- generating redox-cycling process might cause NADPH/H$^+$ exhaustion, which may lead to insufficient sulfite reduction and in turn could trigger a sulfur limitation response. On the other hand, impairment of cysteine biosynthesis by O_2^- stress in *E. coli* is correlated with damage to the cell envelope and subsequent leak of sulfite (Benov et al., 1996). These results were

obtained using a O_2^- accumulating *sodA* mutant, allowing the exclusion of secondary effects. Many bacteria produce low-molecular-mass thiols for protection against oxygen toxicity, e.g. glutathione or mycothiol (Newton et al., 1996). In *B. subtilis*, a comparable mechanism was not described up to now. However, a protective function of low-molecular-mass thiols from sulfur sources under oxidative stress conditions, especially after O_2^- stress, cannot be finally excluded. The increased requirement of such compounds could therefore also explain the observed enhanced expression of genes encoding proteins involved in sulfur assimilation and synthesis of the sulfur-containing amino acids after exposure to paraquat.

SOS regulon. The genes belonging to the SOS regulon are controlled by the RecA/LexA (RecA/DinR) regulators (Miller et al., 1996). Members of this regulon which encode the DNA-damage inducible (Din) proteins are transcriptionally induced after DNA damage (Love et al., 1985). Nearly half of these genes exhibited significantly increased mRNA amounts after exposure to H_2O_2. The induction of e.g. *recA*, *lexA*, *uvrABC* and *dinC* (renamed *tagC* in the SubtiList database) suggests H_2O_2 induced DNA damage and subsequent RecA/LexA activation under the chosen conditions. Several of the other known members of the *din* regulon exhibited elevated expression but missed the criterion of significance. The *yneABynzC* operon also belongs to the SOS regulon of *B. subtilis* and was recently found to be induced after H_2O_2 challenge (Kawai et al., 2003). The *yneB* gene exhibited one of the strongest induction factors after peroxide treatment detected in this study, without a concomitant higher induction by paraquat. While YneA represents the functional counterpart of the cell division inhibitor SulA of *E. coli*, YneB shares similarities to resolvases (Kunst et al., 1997; Kawai et al., 2003).

Interestingly, increased expression of members of the SOS regulon was mostly limited to the generation of oxidative stress by addition of H_2O_2, and was much less pronounced after paraquat treatment. This finding might be explained by a strictly controlled dismutation of O_2^- after paraquat addition which is followed by efficient catalase dependent detoxification of the generated H_2O_2. Thus, generation of hydroxyl radicals from O_2^- might be kept to a minimum after paraquat treatment. In contrast, H_2O_2, which might be available in much higher amounts after peroxide addition for a short period of time, could be partly converted to $OH^•$ via Fenton chemistry, resulting in larger amounts of this highly DNA-damaging agent.

Stringent regulated genes. Preliminary data indicate that oxidative stress induces ppGpp accumulation in *E. coli* (VanBogelen et al., 1987). In our study, the majority of the genes which were previously identified to be up- or downregulated in the course of the stringent response induced by amino acid limitation (Eymann et al., 2002) also responded to oxidative stress. Positively stringent-regulated genes encoding extracellular serine proteases (Epr, Vpr) and urease (UreABC) were found at elevated expression levels after one or both stimuli. Furthermore, both ROS effected the downregulation of genes encoding ribosomal proteins and translation factors, indicating an extensive downregulation of the protein synthesis apparatus. However, O_2^- stress provoked stronger effects compared to H_2O_2 stress. This more pronounced response to O_2^- stress most probably results from the strong growth inhibition caused by the addition of paraquat. Whereas H_2O_2 is assumed to be rapidly degraded by cellular detoxification mechanisms, the continuous generation of O_2^- by the redox-cycling agent paraquat may cause an extreme overload of the cellular capability to cope with oxidative stress. Most probably, these differences in the extent of the oxidative stress are responsible for the observed differences in the growth behavior and the intensity of the stringent response activation.

Other genes upregulated by oxidative stress. Besides several genes encoding products without known function, the expression of genes encoding proteins with predicted roles as antioxidants was also found to be increased in response to both stimuli. Clearly antioxidant functions are assigned to the gene products of *trxA*, *trxB*, *msrA* and *tpx*. The thioredoxin TrxA and the corresponding reductase TrxB are suggested to function in defense against oxidative stress by directly detoxificating H_2O_2 (Spector et al., 1988), acting as hydrogen donor for peroxidases (Chae et al., 1994a) or by reactivating oxidatively damaged proteins, notably with non-native disulfide bonds (Fernando et al., 1992). The peptidyl methionine sulfoxide reductase MsrA is predicted to reduce methionine sulfoxides to methionine, thus restoring protein function after oxidative stress in *B. subtilis*, analogous to the role of MsrA in *E. coli* (Moskovitz et al., 1995). The *tpx* gene encodes a predicted thiol peroxidase that probably act in peroxide detoxification. Furthermore, genes which encode proteins with similarities to NADH-dependent flavin oxidoreductases (*yqiG, yqjM*) and several dehydrogenases exhibited increased expression after H_2O_2 and after paraquat challenge. It was recently reported that the YqjM protein shares similarity with the yeast Old Yellow Enzyme and is rapidly induced by addition of H_2O_2 or trinitrotoluene

suggesting a role in detoxification (Fitzpatrick et al., 2003). The upregulation of a putative nitro/flavin reductase encoded by $nfrA$ after H_2O_2 and paraquat treatment indicates a function in the reduction of nitric oxides after oxidative stress (Zenno et al., 1998; Kobori et al., 2001). In *E. coli*, the $nfrA$ homologue $nfsA$ is induced by O_2^- in a SoxRS-dependent manner (Benov & Fridovich, 2002).

About 30 genes of unknown function exhibited significantly elevated expression levels after paraquat treatment and were less or not at all induced after H_2O_2 challenge. Only a weak induction of the $sodA$ gene encoding the Mn-dependent superoxide dismutase was detectable after O_2^- stress. The homologous gene in *E. coli* was demonstrated to be strongly induced in a SoxRS-dependent manner (Pomposiello & Demple, 2002). Two *B. subtilis* open reading frames ($sodF$ and $yojM$) encoding proteins with similarities to Fe- and CuZn-dependent superoxide dismutases of *E. coli* (Kunst et al., 1997) were not induced after paraquat treatment. The spontaneous dismutation of O_2^- is supposed to be a very slow process. For efficient detoxification, conversion of O_2^- to H_2O_2 should be based on an antioxidant activity (Fridovich, 1995; Fridovich, 1997). Within the group of antioxidants, manganese, Mn(II) complexes and manganous porphyrins are capable of eliminating O_2^- (Archibald & Fridovich,

1981; Archibald & Fridovich, 1982). The relatively high amount of manganese in our growth medium may serve for O_2^- dismutation under the chosen conditions, thus making an induction of Superoxide dismutase unnecessary. This is in accordance with studies of Inaoka et al. (1999), who reported Mn(II)-dependent O_2^--scavenging activity after paraquat treatment in *B. subtilis*. However, no induction of intracellular superoxide dismutase activity was found with or without manganese after paraquat exposure by these authors (Inaoka et al., 1998).

Several genes were found to be significantly upregulated following H_2O_2 exposure but to a lesser extent or not at all after paraquat challenge. For most of their products, the function in peroxide resistance, if any, is still unknown.

Genes downregulated by oxidative stress. The datasets for all genes significantly downregulated by peroxide and paraquat stress including all expression level ratios are available as supplementary data with the online version of this paper at http://mic.sgmjournals.org. In addition to the stringent response (see above), both stimuli caused significant repression of genes encoding proteins involved in the biosynthesis of purines, pyrimidines, arginine and histidine. Furthermore, several genes which encode enzymes of glycolysis

and tricarboxic acid cycle were downregulated. The finding that the expression of several genes was strongly decreased by paraquat treatment but only slightly or not at all by H_2O_2 challenge may be due to the stronger growth inhibition after paraquat exposure. In contrast, several genes were only downregulated after peroxide challenge, among them the members of the *cysH* and the *srf* operon.

Proteins up- or downregulated by oxidative stress. The results of the 2D PAGE analyses after H_2O_2 and paraquat treatment (Fig. 1A, B), were in good agreement with these obtained in the transcriptome analyses. While members of the PerR, Fur and CtsR regulons were found to be induced by both stimuli (e.g. KatA, AhpC, AhpF, MrgA; DhbB, DhbD; ClpC), proteins encoded by σ^B- and RecA-regulated genes were exclusively or more distinct upregulated after H_2O_2 challenge (e.g. GsiB; RecA). The synthesis of the methionine synthase MetE was reduced after peroxide stress but continued after exposure to paraquat. Both stimuli caused an extensive downregulation of the protein synthesis apparatus. A huge number of vegetative proteins were no more or less synthesized after the exposure to peroxide or paraquat, e.g. Ef-Tu, Ef-G, PtsH, PdhA and PurB. This downregulation of the protein biosynthesis apparatus was more pronounced after O_2^- challenge compared to exposure to H_2O_2.

Concluding remarks. Exposure of *B. subtilis* cells to oxidative stress causes major changes in the global gene expression pattern. Besides partially overlapping responses induced by H_2O_2 and paraquat, clearly stimulus-specific gene expression patterns were detected. Both stimuli strongly induced the PerR-dependent stress response, albeit to a different extent. Furthermore, derepression of the Fur regulon, induction of proteins with antioxidant functions and a slight activation of the CtsR regulon after both stimuli was observed. The negatively stringent controlled genes were also downregulated by both stresses, although the response was more distinct after addition of paraquat. The SOS-response was found to be activated primarily by H_2O_2, which also induced a slight σ^B-dependent stress response. Genes encoding proteins involved in sulfur-assimilation and the biosynthesis of cysteine and methionine were solely induced by paraquat challenge, indicating sulfur-limitation. Only a weak induction of *sodA* was detected by the transcriptome and proteome studies, recommending a further analysis of the SodA function in oxidative stress resistance. Our data support the assumption that induction of the

PerR regulon represents the primary stress response after inorganic peroxide stress, whereas the weak induction of the σ^B regulon in the absence of additional limitations or stimuli is of minor physiological importance. No induction of the OhrR regulated *ohrA* (*yklA*) gene was observed after H_2O_2 treatment, emphasizing the previously reported function of this gene in organic peroxide resistance but not in H_2O_2 stress resistance (Fuangthong et al., 2001).

The combination of transcriptome and proteome analysis established a panoramic view on the adaptational strategies of *B. subtilis* to oxidative challenge. Many genes with still unknown function were observed to be induced or repressed by one or both stimuli. The next step in the context of a systematic analysis should be to address the contribution of the products encoded by the induced genes to oxidative stress defense, with the goal of a comprehensive view on stress adaptation after exposure to ROS.

Table 1. Genes upregulated after addition of H_2O_2 (H) or paraquat (P) as revealed by transcriptome and proteome analysis.

For the transcriptome analysis, arithmetic mean and standard deviation calculated from the four expression level ratios per gene and condition are given. Gene names and expression ratios in boldface indicate significant upregulation at the mRNA level under at least one stress condition. Significant upregulation means that at least three of the four expression level ratios determined per condition are ≥ 3. For the proteome analysis, increased protein synthesis after addition of H_2O_2, paraquat, or both, as revealed by the corresponding proteome analysis is indicated by '+'. The information given in the column 'Gene product function' is according to the SubtiList Database (http://genolist.pasteur.fr/SubtiList/). Horizontal lines in the body of the table define the transcriptional units.

Gene	Transcriptome				Proteome		Gene product function
	H		P		H	P	
PerR-regulated genes							
ahpC	**17.3**	± 11.5	**18.4**	± 8.5	+	+	Alkyl hydroperoxide reductase (small subunit)
ahpF	**9.3**	± 0.8	**9.8**	± 3.2	+	+	Alkyl hydroperoxide reductase (large subunit)
fur	**4.1**	± 0.6	**4.0**	± 1.9			Transcriptional repressor of iron uptake
hemA	**3.9**	± 0.8	3.6	± 1.2			Glutamyl-tRNA reductase
hemX	**3.5**	± 0.9	2.0	± 0.5			Negative effector of the concentration of HemA
hemC	**3.8**	± 0.9	1.8	± 0.7			Porphobilinogen deaminase
hemD	3.4	± 0.6	1.8	± 0.3			Uroporphyrinogen III cosynthase
hemB	2.4	± 0.3	2.0	± 0.8			Delta-aminolevulinic acid dehydratase
hemL	3.0	± 0.7	1.7	± 0.4			Glutamate-1-semialdehyde 2,1-aminotransferase
katA	**145.8**	± 49.3	**171.2**	± 69.2	+	+	Vegetative catalase 1
mrgA	**85.8**	± 33.1	**163.6**	± 24.2	+	+	Metalloregulation DNA-binding stress protein
perR	2.6	± 0.4	**3.9**	± 0.6			Transcriptional repressor of the peroxide regulon
yoeB	1.1	± 0.4	**13.0**	± 2.9			Unknown
yuiA	1.9	± 0.6	**3.6**	± 1.0			Similar to unknown proteins from *B. subtilis*
yuiB	2.7	± 0.5	**7.1**	± 1.0			Similar to unknown proteins from *B. subtilis*
yxbC	2.1	± 0.6	**4.1**	± 0.9			Similar to unknown proteins
zosA	**9.3**	± 1.3	**18.4**	± 2.8			Similar to heavy metal-transporting ATPase
Fur-regulated genes							
dhbA	**11.1**	± 3.4	**11.1**	± 3.8		+	2,3-Dihydro-2,3-dihydroxybenzoate dehydrogenase
dhbC	**4.6**	± 1.2	**4.6**	± 1.1	+	+	Isochorismate synthase
dhbE	**8.1**	± 3.3	**5.4**	± 1.8	+	+	2,3-Dihydroxybenzoate-AMP ligase
dhbB	**32.4**	± 7.1	**23.2**	± 7.5	+	+	Isochorismatase
dhbF	**7.6**	± 1.6	**7.3**	± 4.7			Involved in 2,3-dihydroxybenzoate synthesis
fhuD	**4.8**	± 0.5	3.7	± 1.1			Ferrichrome ABC transporter (ferrichrome-binding protein)
gabD	**8.7**	± 3.1	**11.1**	± 2.2			Succinate-semialdehyde dehydrogenase
ypbR	**3.1**	± 0.5	1.5	± 0.1			Similar to unknown proteins
ydbN	**5.8**	± 2.7	**6.5**	± 2.3			Unknown
yfhC	2.7	± 0.4	**3.1**	± 0.5			Similar to unknown proteins
yhfP	**3.6**	± 1.0	2.3	± 0.6			Similar to unknown proteins
ykuN	**4.1**	± 0.7	**5.5**	± 0.6			Similar to flavodoxin
ykuO	**18.2**	± 10.8	**21.6**	± 6.7			Unknown

Table 1. genes upregulated cont.

Gene	Value 1	±	Value 2	±			Description
ykuP	**50.8**	± 16.3	**42.7**	± 11.9			Similar to flavodoxin
yuiI	**11.1**	± 1.0	**4.0**	± 1.0			Similar to unknown proteins
ywbL	1.7	± 0.2	1.5	± 0.3			Similar to unknown proteins
ywbM	**5.1**	± 1.6	**3.5**	± 0.5			Similar to unknown proteins
ywbN	**4.0**	± 1.2	1.9	± 0.9			Similar to unknown proteins
yxeB	**14.2**	± 4.9	**9.3**	± 3.1			Similar to ABC transporter (binding protein)
σ^B-regulated genes							
csbD	**3.6**	± 1.3	2.3	± 0.5			σ^B-controlled gene
gsiB	**6.7**	± 3.2	**3.1**	± 1.2	+		General stress protein
gspA	**9.4**	± 4.7	3.0	± 0.5	+		General stress protein
ycdF	**4.5**	± 2.2	1.6	± 0.4			Similar to glucose 1-dehydrogenase
ycdG	2.0	± 0.8	1.8	± 0.4			Similar to oligo-1,6-glucosidase
ydaP	**3.7**	± 1.5	1.5	± 0.4			Similar to pyruvate oxidase
yflT	**8.9**	± 7.4	2.1	± 0.8			Unknown
yjgC	**7.3**	± 3.2	2.0	± 0.5			Similar to formate dehydrogenase
yjgD	**8.0**	± 7.2	2.0	± 1.1			Similar to unknown proteins from *B. subtilis*
yraA	**5.1**	± 1.7	**10.8**	± 1.6			Similar to general stress protein
ytxG	2.8	± 1.0	2.2	± 0.5			Similar to general stress protein
ytxH	**3.0**	± 0.8	**2.9**	± 0.5			Similar to general stress protein
ytxJ	2.9	± 0.3	2.5	± 0.5			Similar to general stress protein
yvyD	2.5	± 1.3	**3.8**	± 0.7		+	Similar to σ^{54} modulating factor of gram-negative bacteria
ywzA	**3.7**	± 0.7	2.0	± 0.5			Similar to unknown proteins from *B. subtilis*
yxaB	**2.9**	± 0.4	2.7	± 0.5			Similar to unknown proteins
yvgN	**7.8**	± 1.5	**8.8**	± 1.9			Similar to dehydrogenase
CtsR-regulated genes							
ctsR	3.0	± 0.7	2.9	± 0.7			Transcriptional repressor of class III stress genes
mcsA	2.7	± 0.4	**3.4**	± 0.4			Modulator of CtsR repression
mcsB	**4.5**	± 1.2	**4.6**	± 1.0			Modulator of CtsR repression
clpC	**4.1**	± 1.2	**3.7**	± 1.0	+	+	Class III stress response-related ATPase
radA	**4.2**	± 1.3	2.8	± 0.6			DNA repair protein homolog
yacK	3.5	± 1.5	2.3	± 0.8			Similar to unknown proteins
Sulfur-limitation-regulated genes							
cysH	0.5	± 0.1	**5.5**	± 0.8			Phosphoadenosine phosphosulfate reductase
cysP	0.2	± 0.0	**4.0**	± 1.0			Sulfate permease
sat	0.3	± 0.1	**3.5**	± 0.8			Probable sulfate adenylyltransferase
cysC	0.3	± 0.1	3.3	± 1.4			Probable adenylylsulfate kinase
ylnD	0.4	± 0.1	**4.0**	± 0.3			Similar to uroporphyrin-III C-methyltransferase
ylnE	0.5	± 0.0	3.6	± 1.6			Similar to unknown proteins
ylnF	0.5	± 0.1	**4.6**	± 0.6			Similar to uroporphyrin-III C-methyltransferase
cysK	1.1	± 0.4	**7.3**	± 2.3			Cysteine synthetase A

Table 1. genes upregulated cont.

metE	0.5	± 0.0	**4.2**	± 0.9	+	Cobalamin-independent methionine synthase
ssuB	1.5	± 0.5	**9.1**	± 1.5		Aliphatic sulfonate ABC transporter (binding protein)
ssuA	1.0	± 0.2	**4.7**	± 1.8		Aliphatic sulfonate ABC transporter (binding lipoprotein)
ssuC	1.3	± 0.3	**5.6**	± 0.8		Aliphatic sulfonate ABC transporter (permease)
ssuD	1.7	± 0.8	2.7	± 1.4		Aliphatic sulfonate monooxygenase
ygaN	1.2	± 0.2	1.5	± 0.2		Unknown
yitJ	1.0	± 0.3	**6.0**	± 1.6		Similar to unknown proteins
yjcI	1.3	± 0.4	**8.7**	± 1.4		Similar to cystathionine gamma-synthase
yjcJ	0.9	± 0.5	**7.7**	± 1.8		Similar to cystathionine beta-lyase
ykrW	0.9	± 0.1	**3.7**	± 0.7		Similar to ribulose-bisphosphate carboxylase
ykrX	0.9	± 0.0	**4.0**	± 0.4		Similar to unknown proteins
ykrY	0.7	± 0.1	3.1	± 1.0		Similar to unknown proteins
ykrZ	0.7	± 0.1	2.7	± 0.3		Similar to unknown proteins
yoaD	0.9	± 0.2	**4.8**	± 1.2		Similar to phosphoglycerate dehydrogenase
yoaC	0.8	± 0.2	**5.7**	± 2.0		Similar to xylulokinase
yoaB	0.8	± 0.1	**4.7**	± 1.0		Similar to alpha-ketoglutarate permease
yrrT	0.9	± 0.1	**5.0**	± 1.1		Unknown
mtn	0.7	± 0.1	**6.4**	± 1.2		Methylthioadenosine nucleosidase
yrhA	1.1	± 0.2	**28.5**	± 5.9		Similar to cysteine synthase
yrhB	1.2	± 0.1	**19.2**	± 3.2		Similar to cystathionine gamma-synthase
ytlI	1.4	± 0.8	**9.4**	± 1.7		Similar to transcriptional regulator (LysR family)
ytmI	1.3	± 0.4	**8.6**	± 1.9		Similar to unknown proteins from *B. subtilis*
ytmJ	1.0	± 0.2	**7.2**	± 3.4		Similar to amino acid ABC transporter (binding protein)
ytmK	1.0	± 0.2	3.8	± 1.2		Similar to amino acid ABC transporter (binding protein)
ytmL	1.0	± 0.2	3.1	± 0.5		Similar to amino acid ABC transporter (permease)
ytmM	1.0	± 0.2	**3.1**	± 0.5		Similar to amino acid ABC transporter (permease)
ytmN	1.3	± 0.4	3.0	± 0.3		Similar to amino-acid ABC transporter (ATP-binding protein)
ytmO	1.0	± 0.1	**5.9**	± 0.7		Similar to monooxygenase
ytnI	1.3	± 0.6	**3.9**	± 0.7		Unknown
ytnJ	1.0	± 0.2	2.7	± 0.6		Similar to nitrilotriacetate monooxygenase
ribR	0.9	± 0.6	0.9	± 0.2		Riboflavin kinase
hipO	1.2	± 0.2	2.5	± 0.6		Similar to aminohydrolase
ytnM	1.0	± 0.2	1.4	± 0.3		Similar to unknown proteins
yxeK	0.9	± 0.2	**17.3**	± 4.8		Similar to monooxygenase
yxeL	0.8	± 0.3	**20.9**	± 5.6		Similar to unknown proteins from *B. subtilis*
yxeM	0.8	± 0.2	**15.8**	± 4.0		Similar to amino acid ABC transporter (binding protein)
yxeN	1.2	± 0.3	2.8	± 0.2		Similar to amino acid ABC transporter (permease)
yxeO	0.9	± 0.3	**22.0**	± 7.1		Similar to amino acid ABC transporter (ATP-binding protein)
yxeP	1.2	± 0.3	**9.2**	± 2.1		Similar to aminoacylase

Table 1. genes upregulated cont.

Gene						Description
yxeQ	1.1	± 0.2	**5.9**	± 1.6		Similar to unknown proteins
yxeR	1.3	± 0.5	2.5	± 0.9		Similar to ethanolamine transporter
SOS-Regulon						
recA	**8.0**	± 2.6	**3.0**	± 0.8	+	Protein involved in homologous recombination and DNA repair (LexA-autocleavage)
lexA	**4.8**	± 1.9	1.9	± 0.6		Transcriptional repressor of the SOS regulon
uvrB	**4.4**	± 1.2	1.5	± 0.3		Excinuclease ABC (subunit B)
uvrA	**10.5**	± 1.9	2.3	± 0.2		Excinuclease ABC (subunit A)
uvrC	**5.0**	± 1.0	**4.4**	± 1.5		Excinuclease ABC (subunit C)
dinB	**8.8**	± 2.1	3.1	± 1.2		Nuclease inhibitor
tagC (*dinC*)	**6.6**	± 1.2	3.4	± 2.1		Possibly involved in polyglycerol phosphate teichoic acid biosynthesis
yhaO	**3.9**	± 0.7	1.7	± 0.2		Similar to unknown proteins
yhaN	**6.5**	± 2.1	1.4	± 0.4		Similar to unknown proteins
yhaM	2.5	± 0.1	1.1	± 0.1		Similar to CMP-binding factor
yneA	**18.8**	± 3.9	3.7	± 1.1		Unknown
yneB	**64.9**	± 9.5	**9.4**	± 2.2		Similar to resolvase
ynzC	**10.9**	± 3.4	1.2	± 0.3		Similar to unknown proteins
Positively stringent-regulated genes						
epr	1.5	± 0.4	**4.5**	± 0.9		Minor extracellular serine protease
gamP	1.5	± 0.7	**3.7**	± 0.6		Probable PTS glucosamine-specific enzyme IICBA component
rapA	2.3	± 0.8	3.8	± 1.4		Response regulator aspartate phosphatase
phrA	2.5	± 0.6	**4.2**	± 1.3		Phosphatase (RapA) inhibitor
rapC	1.9	± 0.5	2.3	± 1.1		Response regulator aspartate phosphatase
phrC	2.2	± 0.3	**3.6**	± 1.2		Phosphatase (RapC) regulator / competence and sporulation stimulating factor (CSF)
ureA	2.8	± 0.5	**4.6**	± 1.5		Urease (gamma subunit)
ureB	**4.5**	± 0.7	**6.6**	± 0.7		Urease (beta subunit)
ureC	1.6	± 0.2	1.9	± 0.2		Urease (alpha subunit)
vpr	2.6	± 0.4	**3.2**	± 0.4		Minor extracellular serine protease
ypiA	3.1	± 1.5	4.2	± 1.3		Similar to unknown proteins
ypiB	2.1	± 0.3	**4.4**	± 1.8		Similar to unknown proteins
ytzB	**6.3**	± 1.7	**6.3**	± 0.7		Similar to unknown proteins
ytzE	2.8	± 0.4	**4.6**	± 0.8		Similar to transcriptional regulator (DeoR family)
Other genes						
amhX	**3.4**	± 0.4	**4.5**	± 0.8		Amidohydrolase
bsaA	**3.5**	± 0.4	2.5	± 0.6		Putative glutathione peroxidase
ypgQ	3.8	± 1.4	2.1	± 1.0		Similar to unknown proteins
ypgR	3.0	± 0.5	2.4	± 0.4		Similar to unknown proteins
dhaS	2.7	± 0.4	**4.1**	± 0.6		Aldehyde dehydrogenase

Table 1. genes upregulated cont.

guaD	1.2	± 0.2	4.8	± 0.9			Guanine deaminase
hemE	3.5	± 1.4	3.0	± 1.0			Uroporphyrinogen III decarboxylase
hemH	4.6	± 1.5	5.1	± 0.9			Ferrochelatase
hemY	3.8	± 1.4	3.5	± 0.9			Protoporphyrinogen IX and coproporphyrinogen III oxidase
ispA	1.4	± 0.2	4.2	± 0.8			Major intracellular serine protease
lonA	3.1	± 1.0	2.6	± 1.0	+	+	Class III heat shock ATP-dependent protease
ysxC	3.3	± 0.6	3.3	± 0.7			Similar to GTP-binding protein
luxS	1.6	± 0.4	3.4	± 0.4			Probable autoinducer-2 production protein
mecA	1.7	± 0.3	3.5	± 0.5			Negative regulator of competence
msrA	5.2	± 1.3	7.5	± 2.6			Peptidyl methionine sulfoxide reductase
yppQ	1.3	± 0.2	1.8	± 0.2			Similar to peptide methionine sulfoxide reductase
nfrA	8.2	± 2.1	11.2	± 4.2			FMN-containing NADPH-linked nitro/flavin reductase
proH	2.3	± 0.9	4.0	± 0.8			Pyrroline-5-carboxylate reductase
proJ	1.9	± 0.8	1.5	± 0.3			Glutamate 5-kinase
sodA	3.9	± 0.9	3.2	± 0.9	+	+	Superoxide dismutase
tpx	3.8	± 1.0	3.5	± 0.6			Probable thiol peroxidase
trxA	5.7	± 0.4	8.8	± 2.2	+	+	Thioredoxin
trxB	4.0	± 1.5	9.5	± 2.2	+	+	Thioredoxin reductase
ybgA	1.8	± 0.2	4.4	± 0.4			Similar to transcriptional regulator (GntR family)
ycgJ	37.0	± 10.4	1.3	± 0.3			Similar to unknown proteins
ydbM	1.6	± 0.6	6.8	± 1.2			Similar to butyryl-CoA dehydrogenase
yetG	9.1	± 1.8	11.8	± 2.1			Similar to unknown proteins
yfjR	2.3	± 0.1	4.1	± 0.5			Similar to 3-hydroxyisobutyrate dehydrogenase
yhaA	4.1	± 0.7	5.8	± 0.8			Similar to aminoacylase
yhaZ	13.0	± 7.0	3.2	± 1.3			Similar to DNA alkylation repair enzyme
yhbl	5.0	± 0.9	0.7	± 0.1			Similar to transcriptional regulator (MarR family)
yhbJ	4.7	± 0.5	1.0	± 0.1			Unknown
yhcW	2.8	± 0.8	1.7	± 0.3			Similar to phosphoglycolate phosphatase
yhcX	3.9	± 1.2	2.2	± 0.5			Similar to unknown proteins
yhfl	3.7	± 0.5	1.7	± 0.5			Similar to unknown proteins
yhfJ	4.3	± 0.4	2.1	± 0.4			Similar to lipoate-protein ligase
yhfK	3.9	± 0.3	4.1	± 0.1			Similar to unknown proteins
yhjP	2.7	± 0.8	2.7	± 0.5			Similar to transporter binding protein
yhzC	2.4	± 0.5	10.8	± 3.6			Unknown
yjbG	2.5	± 0.2	3.8	± 0.9	+	+	Similar to oligoendopeptidase
yjnA	1.1	± 0.7	4.7	± 1.9			Similar to unknown proteins
ykcA	2.7	± 0.8	3.2	± 0.3			Similar to unknown proteins
yktC	3.4	± 1.3	3.2	± 0.8			Similar to inositol monophosphatase
ykzC	5.3	± 1.9	2.5	± 1.0			Unknown

Table 1. genes upregulated cont.

ylbP	2.3	± 0.6	**3.2**	± 0.5	Similar to unknown proteins
ymaC	**3.4**	± 0.4	1.2	± 0.2	Similar to phage-related protein
ymaD	3.2	± 0.7	1.7	± 0.6	Similar to unknown proteins
yneR	4.0	± 2.1	**3.9**	± 1.0	Similar to unknown proteins
yobE	2.6	± 1.0	**4.6**	± 1.5	Similar to general secretion pathway protein
yocS	2.1	± 0.3	**3.7**	± 1.0	Similar to sodium-dependent transporter
yodM	1.8	± 0.6	2.0	± 0.1	Similar to unknown proteins
yodL	2.2	± 0.6	**3.5**	± 0.5	Unknown
ypbH	1.9	± 0.2	**5.6**	± 2.1	Similar to negative regulation of competence MecA homolog
yqiG	**7.0**	± 2.7	**7.8**	± 2.6	Similar to NADH-dependent flavin oxidoreductase
yqjM	**6.1**	± 1.4	**5.2**	± 0.5	Similar to NADH-dependent flavin oxidoreductase
yqkF	**5.7**	± 0.7	**4.1**	± 0.8	Similar to oxidoreductase
ytdI	**3.4**	± 1.0	**4.9**	± 1.1	Similar to unknown proteins
ytpB	**3.1**	± 0.6	2.7	± 0.4	Similar to unknown proteins
yuaE	**5.8**	± 1.8	**6.0**	± 1.3	Unknown
yueE	3.0	± 1.0	**8.8**	± 2.8	Similar to unknown proteins
yugJ	**13.8**	± 4.3	**11.7**	± 3.5	Similar to NADH-dependent butanol dehydrogenase
yugP	2.9	± 0.7	**7.4**	± 1.3	Similar to unknown proteins
yurP	1.3	± 0.1	1.5	± 0.3	Similar to Gln-Frc-6-phosphate transaminase
yurO	1.4	± 0.3	1.3	± 0.3	Similar to multiple sugar-binding protein
yurN	1.8	± 0.4	2.1	± 0.1	Similar to sugar permease
yurM	2.3	± 0.3	2.7	± 0.7	Similar to sugar permease
yurL	2.5	± 0.8	**3.2**	± 0.7	Similar to ribokinase
yusX	**3.2**	± 0.3	2.8	± 0.4	Similar to oligoendopeptidase
yusY	1.9	± 0.4	1.8	± 0.1	Similar to oligoendopeptidase F
yutI	2.3	± 0.9	**3.5**	± 0.8	Similar to NifU protein homolog
yutM	1.9	± 0.2	**2.9**	± 0.4	Similar to unknown proteins
yvdT	1.7	± 0.4	**4.5**	± 0.8	Similar to transcriptional regulator (TetR/AcrR family)
yvdS	1.4	± 0.7	2.3	± 1.0	Similar to molecular chaperone
yvdR	**3.6**	± 0.5	**4.0**	± 0.4	Similar to molecular chaperone
yvrD	**7.3**	± 0.9	**14.6**	± 4.0	Similar to ketoacyl-carrier protein reductase
ywlF	**2.9**	± 0.5	**5.2**	± 0.8	Similar to ribose 5-phosphate epimerase (pentose phosphate)
ywlG	2.7	± 0.2	**4.3**	± 0.8	Similar to unknown proteins
ywnF	**3.8**	± 2.0	**17.7**	± 7.8	Similar to unknown proteins
ywrO	**4.8**	± 0.9	**5.4**	± 2.2	Similar to NAD(P)H oxidoreductase
yybR	1.3	± 0.3	**4.3**	± 1.1	Similar to ester hydrolase

Patterns of Protein Carbonylation following Oxidative Stress in Wild-type and *sigB* Mutant *Bacillus subtilis* Cells

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Summary. Oxidative stress causes damage to nucleic acids, membrane lipids and proteins. One striking effect is the metal-catalyzed, site-specific carbonylation of proteins. In the Gram-positive soil bacterium *Bacillus subtilis*, the PerR-dependent specific stress response and the σ^B-dependent general stress response act together to make cells more resistant to oxidative stress. In this study, we analyzed the carbonylation of cytoplasmic proteins in response to hydrogen peroxide stress in *B. subtilis*. Furthermore, we asked whether the σ^B-dependent response to oxidative stress also confers protection against protein carbonylation. To monitor the amount and specificity of protein damage, carbonyls were derivatized with 2,4-dinitrophenylhydrazine, and the resulting stable hydrazones were detected by immunoanalysis of proteins separated by one- or two-dimensional gel electrophoresis. The overall level of protein carbonylation increased strongly in cells treated with hydrogen peroxide. Several proteins, including the elongation factors EF-G, TufA and EF-Ts were found to be highly carbonylated. Induction of the peroxide specific stress response by treatment with sub-lethal peroxide concentrations, prior to exposure to otherwise lethal levels of peroxide, markedly reduced the degree of protein carbonylation. Cells starved for glucose also showed only minor amounts of peroxide-mediated protein carbonylation compared to exponentially growing cells. We could not detect any differences between wild-type and $\Delta sigB$ cells starved for glucose or preadapted by heat treatment with respect to the amount or specificity of protein damage incurred upon subsequent exposure to peroxide stress. However, artificial preloading with proteins that are normally induced by σ^B-dependent mechanisms resulted in a lower level of protein carbonylation when cells were later subjected to oxidative stress.

Introduction

In populations of *Bacillus subtilis* cells one of the earliest adjustments to stress and starvation involves the synthesis of a large

set of general stress proteins. Heat, salt, ethanol or acid stress, or starvation of glucose, oxygen or phosphate induces the expression of more than 150 genes, which are controlled by the alternative sigma factor σ^B (for review see Price, 2000; Hecker & Völker, 2001; Gaidenko et al., 2002). Besides providing non-specific protection against heat, osmotic and acid stress, phenotypic studies have revealed the general stress response plays a role in resistance to oxidative stress (Hecker & Völker, 1998; Hecker & Völker, 2001). However, the primary defense against the effects of oxygen radicals involves the induction of specific stress proteins, which is controlled by the redox-regulated PerR repressor protein, while resistance to organic hydroperoxides is mediated by OhrR (Chen et al., 1995; Bsat et al., 1998; Fuangthong et al., 2001). After exposure to sub-lethal levels of hydrogen peroxide, detoxification proteins including catalase (KatA), alkylhydroperoxidase (AhpCF) and DNA protectant MrgA are induced by the inactivation of PerR (Bsat et al., 1996). During glucose starvation, B. subtilis cells develop cross-resistance to the radical producing agents cumene hydroperoxide and hydrogen peroxide, while sigB mutant cells partially fail to enhance their resistance under these conditions (Antelmann et al., 1996; Engelmann & Hecker, 1996). Detailed investigations of

the σ^B-regulon have revealed a possible involvement of proteins homologous to detoxifying catalase and DNA protecting proteins induced at the onset of stationary phase in Escherichia coli (Von Ossowski et al., 1991; Almirón et al., 1992; Engelmann et al., 1995; Antelmann et al., 1997a). While the DNA binding protein Dps was found to be essential for the survival of glucose-starved B. subtilis cells treated with H_2O_2, the σ^B-dependent katalase KatE does not seem to be involved in this resistance (Engelmann & Hecker, 1996; Antelmann et al., 1997b).

Oxidative stress is defined as an intracellular surplus of pro-oxidants resulting either from depletion of anti-oxidants or from an increase in levels of reactive oxygen species (Farr & Kogoma, 1991). Reactive oxygen species (ROS) are primarily generated as by-products of normal respiratory activity in aerobically growing bacteria (Imlay & Fridovich, 1991). Exposure to ionizing radiation, redox-active compounds and metals may further enhance the intracellular concentration of ROS, resulting in toxicity if the amounts exceed the capacity of the defense and repair systems present in cells.

All biologically relevant macromolecules, i.e. nucleic acids, membrane lipids and proteins, are susceptible to damage by oxygen radicals. Oxidative modifications of proteins are assumed to impair or even

inhibit a wide range of biochemical functions, resulting in the disruption of metabolic pathways (Stadtman, 1993). Accumulated oxidatively damaged proteins have to be degraded by proteolytic systems (Rivett, 1986; Davies, 1987). The discovery that oxidatively altered forms of proteins appear in the context of aging, oxidative stress and some pathological conditions has focused attention on the characteristics of these modifications (for review see Berlett & Stadtman, 1997). Besides the fragmentation of polypeptide chains, oxidation of proteins leads to modifications of amino acid side chains, e.g. the generation of methionine sulfoxides, cysteine sulfonic acids, cysteine disulfide bonds or tyrosine cross-links. Another striking alteration is the introduction of carbonyl groups in proteins at lysine, arginine, proline or threonine residues by a metal-catalyzed, site-specific reaction (Stadtman, 1993).

Carbonyl moieties in protein side chains are readily detectable by derivatization with 2,4-dinitrophenylhydrazine (DNPH). The corresponding stable protein-bound hydrazone (DNP) can be quantified either by spectrophotometry or by immunodetection using a DNP-specific primary antibody (Levine et al., 1994; Shacter et al., 1994). The level of protein carbonyls can thus serve as an indicator for oxidative protein modification, and permits one to draw conclusions regarding the severity of protein damage in tissue, cell and protein samples.

In this work, we analyzed extracts of *B. subtilis* cells treated with H_2O_2 in order to identify the major protein targets of carbonylation. In addition, we asked whether the σ^B-dependent general stress response is involved in protecting against the formation of carbonyl groups on proteins in cells exposed to oxidative stress.

Methods

Strains, cultivation and stress conditions. All experiments were carried out using the wild-type *B. subtilis* strain 168 (*trpC2*), its isogenic *sigB* mutant ML6 (*trpC2 sigB*::ΔHindIII-EcoRV::*cat*) (Igo et al., 1987) or *B. subtilis* strain BSA115 (*trpC2 rsbU*::*kan* $P_B\Delta28$::P_{spac}*rsbW313* pTET-I SPβ *ctc*::*lacZ*) (Völker et al., 1995a). Bacteria were grown aerobically with vigorous agitation at 37°C in a synthetic medium (Stülke et al., 1993). When appropriate, the medium was supplemented with chloramphenicol (final concentration: 5 μg/ml) or kanamycin (10 μg/ml). Induction of the peroxide specific stress response was achieved by pretreating exponentially growing cells with 50 μM H_2O_2 (Sigma) for 10 min prior to exposure to 10 mM H_2O_2. Activation of the general stress response was accomplished by exposing cells to mild

heat stress or starving them for glucose. For heat treatment, cells were grown to an $OD_{500\,nm}$ value of 0.4 and transferred to 48°C for 15 min. Cells were transferred back to 37°C, and hydrogen peroxide was added immediately to the final concentration desired. For glucose starvation experiments, strains were grown to an $OD_{500\,nm}$ value of 1.0 in synthetic medium with limiting amounts of glucose (0.05% (w/v) glucose). Then 30 min after cells entered stationary phase, H_2O_2 was added. σ^B preloading experiments were performed with *B. subtilis* strain BSA115, in which the *sigB* gene is controlled by an IPTG-inducible promoter and the gene encoding anti-sigma factor RsbW has been deleted. Cells were grown to an $OD_{500\,nm}$ =0.15 and 1 mM IPTG was added. After further 60 min cells were exposed to H_2O_2.

The viability of BSA115 cells before and after exposure to oxidative stress was tested by plating them on LB-plates. Samples were withdrawn at specific time points and diluted. Appropriate dilutions were then plated, incubated at 37°C overnight and the number of colony forming units was determined.

Analysis of protein carbonyl groups. The appearance of carbonyl groups in proteins was analyzed by immunodetection of protein-bound 2,4–dinitrophenylhydrazone

after derivatization with the corresponding hydrazine. Samples were taken at the indicated time points during culture. Cells were harvested by centrifugation and washed once in TE-buffer (10 mM TrisHCl pH 7.5, 1 mM EDTA). Pellets were resuspended in TE-Buffer containing 1 mM PMSF, and crude cell extracts were obtained by disruption using SLM Aminco French Pressure Cell or by sonication. Lysates were centrifuged for 30 min (4°C, 15000 rpm) and the protein concentration in the supernatant was determined using the Roti-Nanoquant protein determination assay (Roth chemicals). To monitor protein carbonyls after one-dimensional (1D) gel electrophoresis, aliquots were mixed with SDS to a final concentration of 6% (w/v). Samples were derivatized with an equal volume of DNPH-derivatization solution (Fluka, 20 mM DNPH in 10% trifluoracetic acid (v/v)) and incubated at room temperature for 15 min. The derivatization reaction was stopped by adding neutralization solution (2M Tris-Base, 30% glycerol (v/v), bromophenolblue). If a derivatization negative control was required, samples were treated with neutralization solution instead of DNPH-derivatization solution. For non-derivatized samples no signal pattern was detectable. Therefore, potentially false positive signals were assumed to be negligible and not further analyzed.

Derivatized protein extracts were analyzed by conventional (1D) polyacrylamide gel electrophoresis (SDS-PAGE), using 25 µg of total protein per lane. Gels were either stained with Coomassie brilliant blue or transferred to PVDF membrane filters (Millipore) using a tank blot system. The filters were either stained with Amido black in advance, or immediately incubated with anti-DNP antibodies (Sigma). Filters were subsequently treated with alkaline-phosphatase conjugated secondary antibodies (Sigma). Bound antibodies were visualized directly by using the chromogenic substrate NBT/BCIP (Roth). When higher detection sensitivity was required, CDP* (Tropix) was used as luminescent substrate and signals were recorded with the Lumi imager system (Roche Applied Science). The specificity of carbonyl group formation in proteins was investigated using two-dimensional (2D) electrophoresis (Isoelectric focusing followed by SDS-PAGE) prior to immunodetection of DNPH. Protein samples were prepared as follows. Crude extracts containing 160 µg of protein were loaded onto IPG strips (pH 4-7, Amersham Pharmacia Biotech) for separation in the first dimension by isoelectric focusing. The DNPH-derivatization reaction was carried out according to the 'in-stripe derivatization protocol' (Reinheckel et al., 2000): The

protein-loaded IPG strips were briefly rinsed with distilled water and incubated in DNPH-derivatization solution for 30 min at room temperature. The reaction was stopped by extensive washing with neutralization solution. The strips were then applied to the second-dimension SDS-PAGE according to Bernhardt et al. (1997). For Western analysis, gels were placed in a semidry blotting system and proteins were electrophoretically transferred to PVDF membrane. Immunodetection was carried out as described above.

For the detection of general stress proteins in 1D assays, antibodies raised against the σ^B-dependent proteins GsiB and Dps were used instead of anti DNP antibodies.

Results

The protein carbonyl content increases after treatment of cells with hydrogen peroxide. When growing *B. subtilis* cells are exposed to hydrogen peroxide concentrations of 50 µM or higher, their growth rate decreases. Exposure of growing cells to 10 mM H_2O_2 leads to cell death with only 0.01-0.1% survival (Murphy et al., 1987). To determine whether proteins in growing cells show evidence of oxidative damage after hydrogen peroxide treatment, the levels of protein carbonyl groups in protein extracts was analyzed using the DNPH immunoassay. Exponentially

growing *B. subtilis* cells were exposed to 1 mM or 10 mM hydrogen peroxide. Samples were taken immediately and 30 min after addition of H_2O_2. Extracts of growing cells showed only weak signals when subjected to the immunoassay. This was taken to represent the basal level of protein carbonylation in growing cells, and was used as a referenz for comparisons with the signal patterns observed after exposing cells to stress. In protein extracts of samples treated with hydrogen peroxide, strong signals were always observed (Fig. 1), the precise pattern of which was dependent on the peroxide concentration used.

Pretreatment of *B. subtilis* cultures with sub-lethal concentrations of H_2O_2 (50 µM) protects cells against the otherwise lethal effect of 10 mM H_2O_2, as revealed by a 10- to 100-fold increase in survival (Murphy et al., 1987). To test the effect at the level of protein damage, cells were pretreated with 50 µM H_2O_2 and then exposed to 10 mM H_2O_2 (Fig. 2). While the carbonyl content was strongly increased in untreated control extracts after oxidative stress, extracts of cells that had first been exposed to the lower dose showed little or no increase in protein carbonylation after incubation for 60 min in the presence of 10 mM H_2O_2.

Upon entry into the stationary phase, *B. subtilis* cells acquire increased resistance to peroxide. Non growing cells
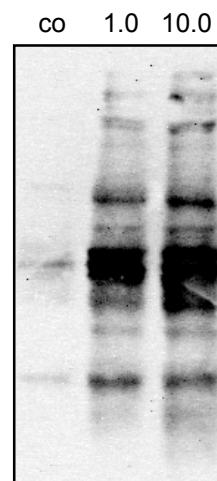
Fig. 1. Monitoring of carbonyl groups in *B. subtilis* cytoplasmic proteins after hydrogen peroxide challenge. Samples were taken before (co) and 30 min after the addition of 1 mM or 10 mM H_2O_2. Oxidatively modified proteins were detected with anti-DNP antibodies after fractionation of DNPH-derivatized proteins by 1D SDS-PAGE.
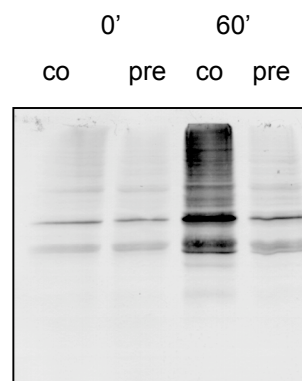

Fig. 2. Patterns of protein carbonylation in *B. subtilis* cells exposed to 10 mM H_2O_2 with (pre) or without (co) prior exposure to a sublethal concentration (50 µM) of H_2O_2. Samples were taken before (0') and 60 min after, 10 mM H_2O_2 was added to the cultures.

that are starved for glucose survive exposures to 10 mM H_2O_2 at rates of nearly 100% (Dowds et al., 1987). To monitor protein carbonyl formation under these conditions, glucose-starved and exponentially growing cells were

challenged with 10 mM hydrogen peroxide for a period of 60 min (Fig. 3). The signal pattern revealed that non-growing cells contain a low levels of protein carbonyls compared to growing cells after peroxide treatment. Furthermore, the signal intensity is only slightly higher than that observed in unstressed cells, indicating that stationary-phase cells are less susceptible to damage by ROS.

Wild-type and *sigB* mutant cells show no differences in level of protein carbonylation. Exhaustion of glucose and mild heat treatment, as well as various other stimuli, induce the σ^B-dependent general stress response in *B. subtilis* cells (Boylan et al., 1993; Völker et al., 1994). Comparisons between *B. subtilis* 168 wild-type and *sigB* mutant cells have revealed a starvation-induced resistance to H_2O_2, which is partially dependent on the general stress response controlled by σ^B (Engelmann & Hecker, 1996). Growing wild-type cells transiently exposed to mild heat stress showed an increased cross-resistance to 0.5 mM H_2O_2, while the *sigB* mutant did not show this effect to the same extent (data not shown). DNPH immunoanalysis was carried out in order to test if the observed resistance to H_2O_2 reflects a σ^B-dependent protection of proteins against oxidative carbonylation. Wild-type and *sigB* mutant cells were
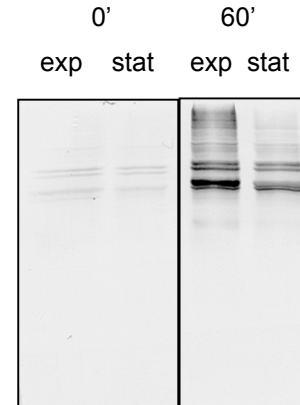
Fig. 3. Detection of protein carbonyls in exponentially growing *B. subtilis* cells (exp.) and non-growing *B. subtilis* cells starved for glucose (stat), after challenge with 10 mM H_2O_2. Samples were taken before (0') and 60 min after 10 mM H_2O_2 was added.
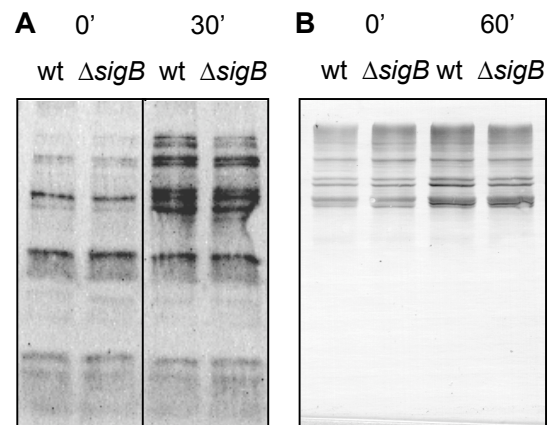

Fig. 4A, B. Comparison of carbonyl group formation in cytoplasmic proteins from wild-type (wt) and σ^B mutant ($\Delta sigB$) *B. subtilis* cells after preadaption followed by oxidative stress. Wild-type and $\Delta sigB$ cells were grown to $OD_{500}=0.4$ and exposed to mild heat stress (48°C) for 15 min (**A**), or to $OD_{500}=1.0$, by which time cells have entered stationary phase because of glucose exhaustion (**B**). Samples were drawn before, and 30 min or 60 min after H_2O_2 challenge. Cytoplasmic protein fractions were prepared and protein carbonyl groups were detected with anti-DNPH antibodies.

preadapted by exposure to non-lethal heat treatment or glucose starvation in order to induce the general stress, and then

subjected to oxidative stress. Cells pre-adapted by heat treatment were exposed to 0.5 mM H_2O_2, and starved cells were treated with 10 mM H_2O_2 (Fig. 4A, B). Comparison between the DNPH signal patterns for proteins from wild-type and *sigB* cells showed no significant differences between them under any of the chosen experimental conditions.

Preloading with general stress proteins results in an increase in resistance to oxidative stress. An artificial preloading system was used to elucidate the impact of the general stress response in the absence of a specific inducing stress, and without exposing cells to harmful environmental stimuli prior to hydrogen peroxide stress (Völker et al., 1995a). In *B. subtilis* strain BSA115, the *sigB* operon is under the control of an IPTG-inducible promoter. In addition, the gene encoding the anti-sigma factor RsbW contains a null mutation. The system enables the IPTG-dependent induction of the general stress response, with the result that general stress protein accumulate (Völker et al., 1999).

Western analysis with anti-GsiB and anti-Dps antibodies were performed to test if σ^B-dependent proteins are indeed synthesized in larger amounts in IPTG-treated BSA115 cells than in wild-type cells exposedto mild stress. The appearance of the σ^B-dependent proteins GsiB and Dps were found to be much higher in IPTG-treated BSA115 cells than in wild-type cells which were exposed to mild heat stress (Fig. 5). Indeed, higher amounts of GsiB and Dps proteins were detected in IPTG-treated BSA115 cells than in wild-type cells of *B. subtilis* that had been exposed to
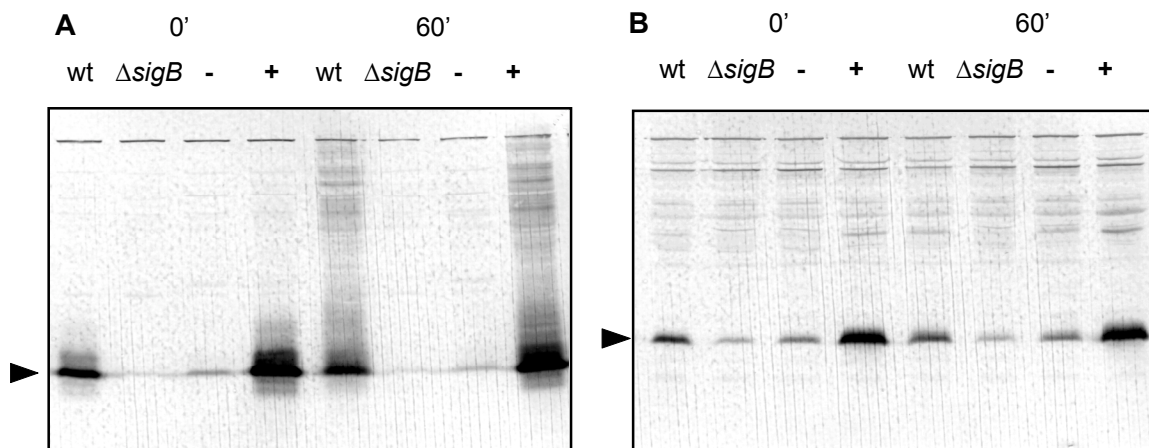

Fig. 5. Accumulation of σ^B-dependent proteins in BSA115 in comparison to wild-type (wt) and *sigB* mutant ($\Delta sigB$) *B. subtilis* cells. When cultures of wt and $\Delta sigB$ cells reached an OD_{500} value of 0.4, cells were exposed to mild heat stress (48°C) for 15 min and than transferred back to 37°C. BSA115 cells were grown to an OD_{500} of 0.15 and subsequently treated (+) or not (-) with 1mM IPTG for 60 min. Samples were taken after heat treatment or IPTG addition and prior to, and 60 min after, H_2O_2 challenge. Western Blots were probed with anti GsiB antibody (**A**) or anti Dps antibody (**B**). The *arrows* indicate the specifically labeled proteins.

lethal concentrations of peroxide. These results indicate that general stress proteins accumulate in BSA115 cells under our experimental conditions.

To examine the development of resistance to hydrogen peroxide as a function of σ^B-activity in BSA115, the cells' ability to survive exposure to the stressor was monitored. Exponentially growing *B. subtilis* BSA115 cells were either treated or not with IPTG for 60 min, followed by exposure to 10 mM hydrogen peroxide for another 60 min. IPTG-treated BSA115 cells, in which σ^B-expression was induced, were up to 1000-fold more resistant to 10 mM H_2O_2 than BSA115 cells that had

not been induced with IPTG before exposure (Fig. 6).

A DNPH immunoassay was carried out to investigate possible differences in the amount of oxidatively modified proteins. IPTG-treated and untreated cells were exposed to 10 mM hydrogen peroxide (Fig. 7). Samples were withdrawn prior to, and 60 min after, exposure to hydrogen peroxide. Proteins extracted from cells not treated with IPTG showed a strong signal pattern indicating intense formation of protein carbonyls. In comparison, IPTG-treated, σ^B-induced, cells showed only a minor increase in protein carbonyls 60 min after exposure to 10 mM H_2O_2. Exposing cultures to lower concentrations of H_2O_2
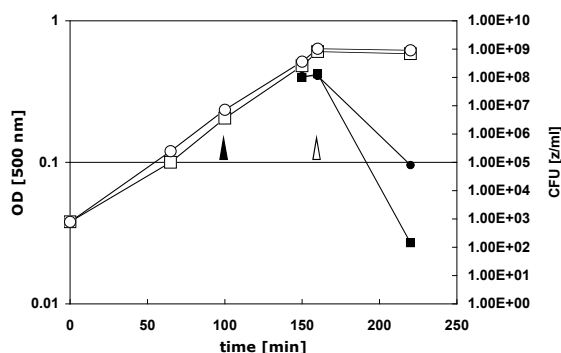

Fig. 6. Influence of the general stress reponse on survival after peroxide challenge. *B. subtilis* BSA115 cells were preloaded with general stress proteins by inducing the expression of σ^B by the addition of IPTG. When the culture density reached OD_{500}=0.15 (indicated by the *filled arrowhead*), 1mM IPTG was added (*open circles*) or not (*open squares*). Sixty minutes later the cells were exposed to oxidative stress by treatment with 10 mM H_2O_2 (*open arrowhead*). Survival was determined as numbers of colony forming units/ml culture by plating appropriate dilutions of samples taken 10 min before, immediatly before, and 60 min after addition of H_2O_2 (*filled circles*, +IPTG; *filled squares*, -IPTG).
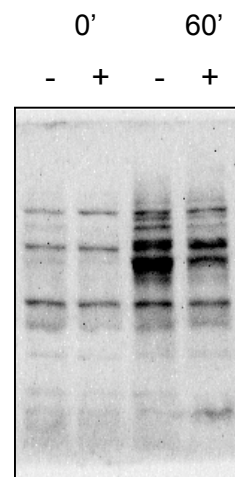

Fig. 7. Detection of protein carbonyls in untreated and σ^B-induced *B. subtilis* BSA115 cells before and after exposure to hydrogen peroxide. Cells were grown to OD_{500} of 0.15 and 1mM IPTG was added (+) or not (-) to exponentially growing cells. After 60 min cultures were exposed to 10 mM H_2O_2. Samples were taken immediately before, and 60 min after, addition of H_2O_2.

resulted in lower signal intensities, with a simultaneous decline in amounts of protein carbonyls in both IPTG-treated and untreated cells.

Two-dimensional immunoassay of protein carbonylation. Two-dimensional SDS-PAGE combined with DNPH immunoanalysis was carried out to examine the specificity of protein carbonylation. Carbonylated proteins were identified by matching the spots to patterns of master gel maps of *B. subtilis* cytoplasmic proteins (Büttner et al., 2001). Several proteins including the elongation factors EF-G (*fusA* gene product), TufA and EF-Ts (*tsf*) showed strong carbonylation signals in growing *B. subtilis* BSA115 cells (Fig. 8A). These proteins are very abundant in growing *B. subtilis* cells. In contrast, other abundant proteins including MetE, IlvC and Hag showed no or only weak signals after DNPH immunodetection, indicating specificity of carbonyl group formation during normal growth. Proteins including PdhD, PdhA, OdhA, SucD, GlnA and ThiC were also observed to be highly carbonylated, but these are present only in small amounts in growing cells. After exposure to 10 mM H_2O_2 for 30 min, an increase in signal intensity was detected for EF-G and TufA (Fig. 8B). Several other proteins, including MetE, Tkt, SdhA, CitA and GlyA, were found to be carbonylated

only under oxidative stress conditions.

To detect possible differences in the specificity of protein carbonylation between untreated and σ^B-induced cells, spot patterns of growing and oxidatively stressed BSA115 cells were compared to those of IPTG-treated BSA115 cells exposed to H_2O_2. Extracts of BSA115 cells grown in the absence of IPTG showed a similar overall signal pattern to growing BSA115 cells treated with IPTG (Fig. 8A, 8C). For a few proteins the corresponding signals could not be found in untreated control extracts and vice versa. The signal patterns of protein extracts exposed to 10 mM H_2O_2 reflected the differences in carbonyl formation already observed with the 1D DNPH immunoassay. Whereas the level of protein carbonylation in σ^B-induced cells remained low, the carbonylation level of IPTG-untreated cells increased significantly (Fig. 8B, D). The pattern of carbonylated proteins in IPTG-treated cells subjected to oxidative stress was very similar to that seen in control extracts.

Discussion

Exponentially growing *B. subtilis* cells are highly sensitive to ROS. This sensitivity was evident after peroxide treatment, when monitoring of carbonyl groups with a DNPH immunoassay revealed high levels of oxidative modification of proteins. 2D DNPH immunoanalysis revealed the selective

nature of carbonylion of *B. subtilis* proteins. In cultures of growing *B. subtilis* cells, elongation factors, TCA cycle enzymes and enzymes involved in amino acid metabolism were found to be carbonylated. Interestingly, one of the amino acid synthetases modified was glutamine synthetase, GlnA. The homologous protein GlnA of *E. coli* was one of the first proteins shown to be damaged by introduction of carbonyl groups (Levine, 1983a; Levine, 1983b). The selectivity of protein carbonylation was previously demonstrated with starving *E. coli* cells by Nyström and coworkers (Dukan & Nyström, 1998; Dukan & Nyström, 1999). 2D immunoanalysis revealed that glutamate synthase, glutamine synthetase, pyruvate kinase and several TCA cycle enzymes are highly susceptible to protein carbonylation in *E. coli* (Dukan & Nyström, 1998). The fact that carbonylation affects common sets of proteins in *E. coli* and *B. subtilis* indicates the presence in these proteins of groups that are readily damaged by oxidative stress.

After exposure to peroxide stress the spot pattern observed by 2D DNPH immunoanalysis of *B. subtilis* revealed several additional targets, while the overall quantity of protein carbonyls increased dramatically. TufA, EF-G and MetE could be identified to be the main targets after hydrogen peroxide treatment. It is not known whether the oxidative damage inhibits the activities inhibition of these proteins after peroxide stress.

Oxidative protein modification in cells reflects a steady state between oxidation events and the rate degradation of oxidized proteins, since protein carbonyls are not repaired (Berlett & Stadtman, 1997). Hence, the accumulation of damaged protein is modulated by the ability of the cells to protect their components, i.e. to activate mechanisms of detoxification, and induce the degradation of damaged proteins in response to destabilizing stimuli. In *B. subtilis*, pretreatment with sub-lethal levels of H_2O_2 leads to higher survival upon subsequent exposure to otherwise lethal concentrations of peroxide (Murphy et al., 1987). Likewise, *B. subtilis* cells treated with 50 µM H_2O_2 before being exposed to 10 mM peroxide showed much less formation of protein carbonyls after oxidative stress than cells that were immediately exposed to lethal peroxide concentrations. These results indicate the involvement of peroxide-specific stress proteins, presumably including the vegetative catalase KatA. KatA has been shown to provide the main defense against peroxide stress in *B. subtilis* cells (Bol & Yasbin, 1990; Engelmann & Hecker, 1996). However, CtsR-dependent induction of the ClpC, ClpE and ClpP protease subunits after oxidative stress suggests that

degradation of oxidized proteins by ATP-dependent proteases also play a role in protecting cells against the effects of peroxide stress (Leichert et al., 2003).

In cells entering the stationary phase because of glucose exhaustion, σ^B-dependent proteins are induced (Völker et al., 1994). The demonstration that (1) stationary phase cells of *B. subtilis* a r e more resistant to hydrogen peroxide than growing wild-type or starving *sigB* mutant cells (Dowds et al., 1987; Engelmann & Hecker, 1996) and (2) that several σ^B-dependent proteins share homologies to proteins involved in protection against oxidative stress (Hecker & Völker, 1998; Petersohn et al., 2001) led us to the suggestion that the general stress response might provide protection against oxidative damage to proteins. DNPH immunoanalysis revealed that neither glucose-starved cells nor cells preadapted by mild heat treatment are protected from protein carbonylation by σ^B upon oxidative stress. However, glucose-starved cells were found to contain lower levels of protein carbonyls after H_2O_2 challenge. Considering that PerR-dependent proteins are induced after glucose exhaustion, stationary phase-dependent protection against protein carbonylation could be a consequence of the action of peroxide specific stress protein (Chen et al., 1995).

When expression of σ^B was activated without concomitant induction of other stress regulons, *B. subtilis* cells were found to be protected against protein carbonylation after peroxide stress. The system used here provides for the accumulation of σ^B-dependent stress proteins to higher levels than those attainable in response to heat stress or glucose starvation. We do not yet know which particular subset of general stress proteins is responsible for the observed protection. Several σ^B-dependent proteins have to be taken into account, including catalase KatE (Engelmann et al., 1995; Engelmann & Hecker, 1996), the DNA-binding and iron-chelating protein Dps (Antelmann et al., 1997b), σ^B-dependent dehydrogenases and perhaps some σ^B-regulated proteases (Petersohn et al., 2001). Whether the observed artificial protection against protein carbonylation after oxidative stress has any ecophysiological significance is, however, a problem to be addressed in future studies.

Fig. 8A-D. Monitoring by 2D DNPH immunoanalysis of the selectivity of protein carbonyl group formation after peroxide stress. BSA115 cells were grown to an OD_{500} of 0.4 and treated with 10 mM H_2O_2. Samples were withdrawn before, and 60 min after peroxide challenge. Carbonylated proteins of growing cells are indicated by *arrows* or *squares* and protein names (**A**). Proteins found to contain new or increased levels of carbonyl groups after exposure to 10 mM H_2O_2 are labeled with *arrows* and protein names or *circles* (unidentified signals), while proteins in which amounts of carbonyls remained constant are labeled in *gray* (**B**). To examine the amount and specificity of carbonylation in cells preloaded with general stress proteins, BSA115 cells were treated with 1mM IPTG and than challenged with 10 mM peroxide. Samples were taken before, and 60 min after, H_2O_2 addition. Proteins of growing σ^B-induced cells showing carbonylation signals are labeled by arrows or squares (**C**). Changes in protein carbonylation after oxidative stress are indicated by *black arrows*, while proteins found to be carbonylated even in growing cells are labeled in gray (**D**).
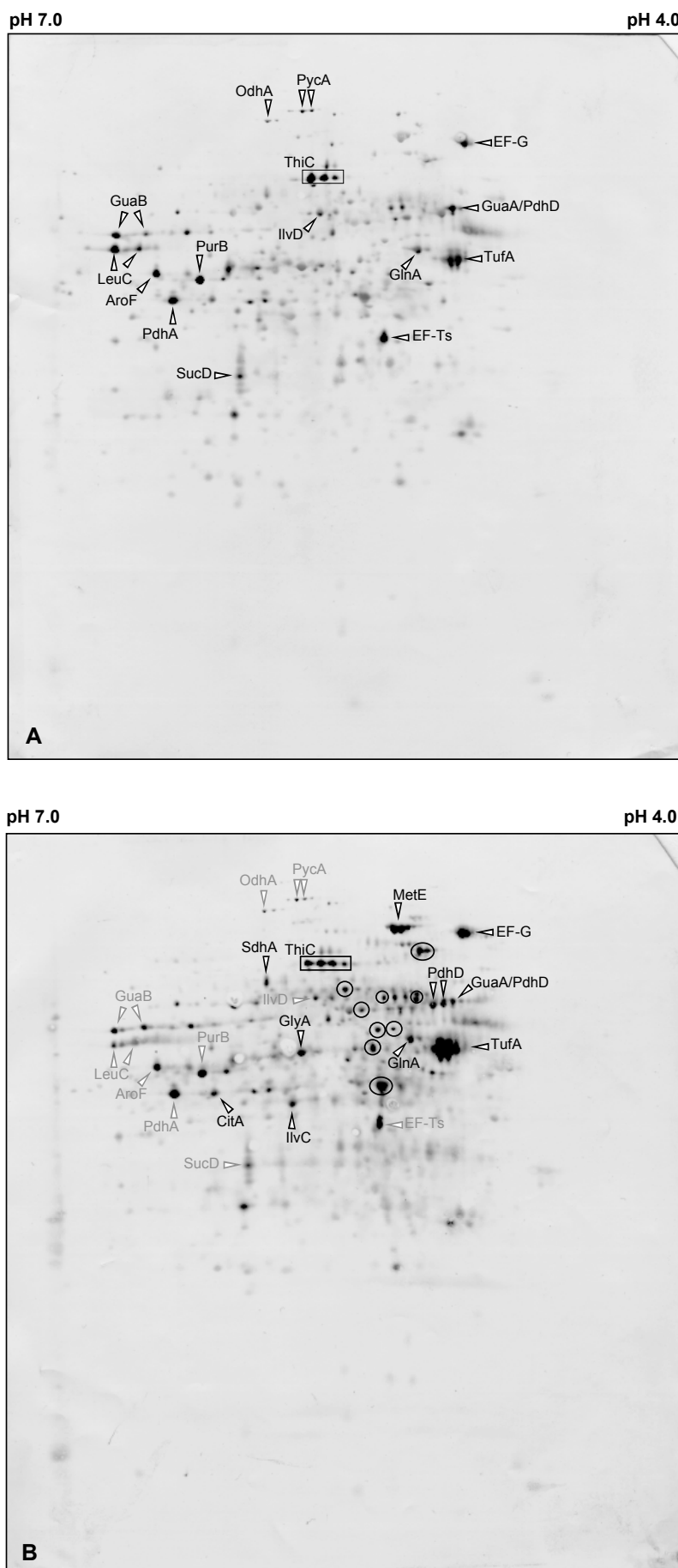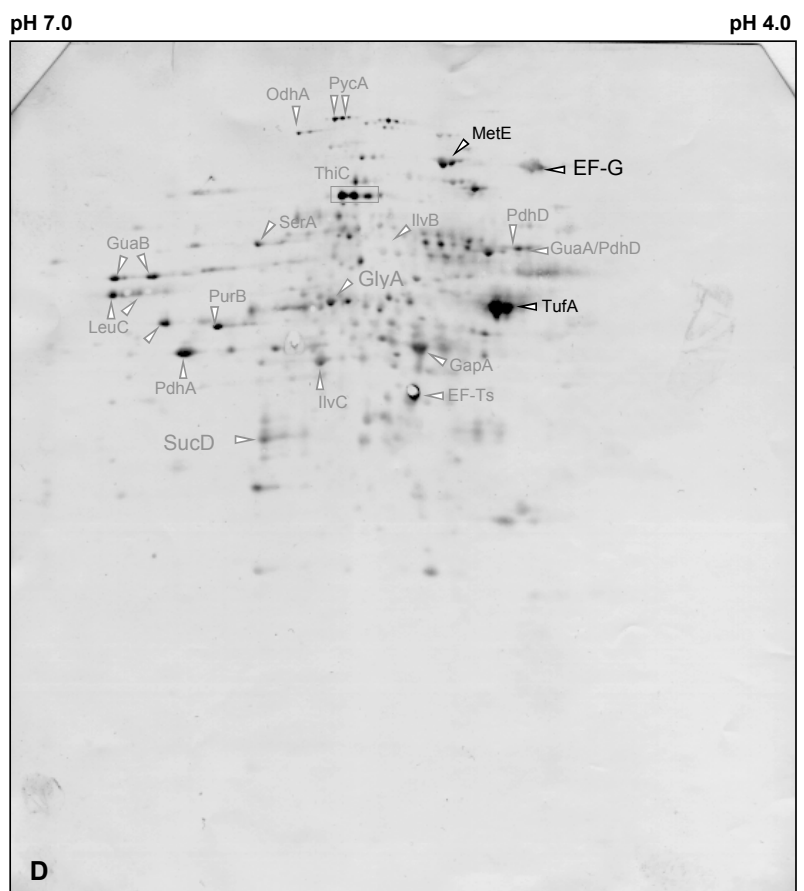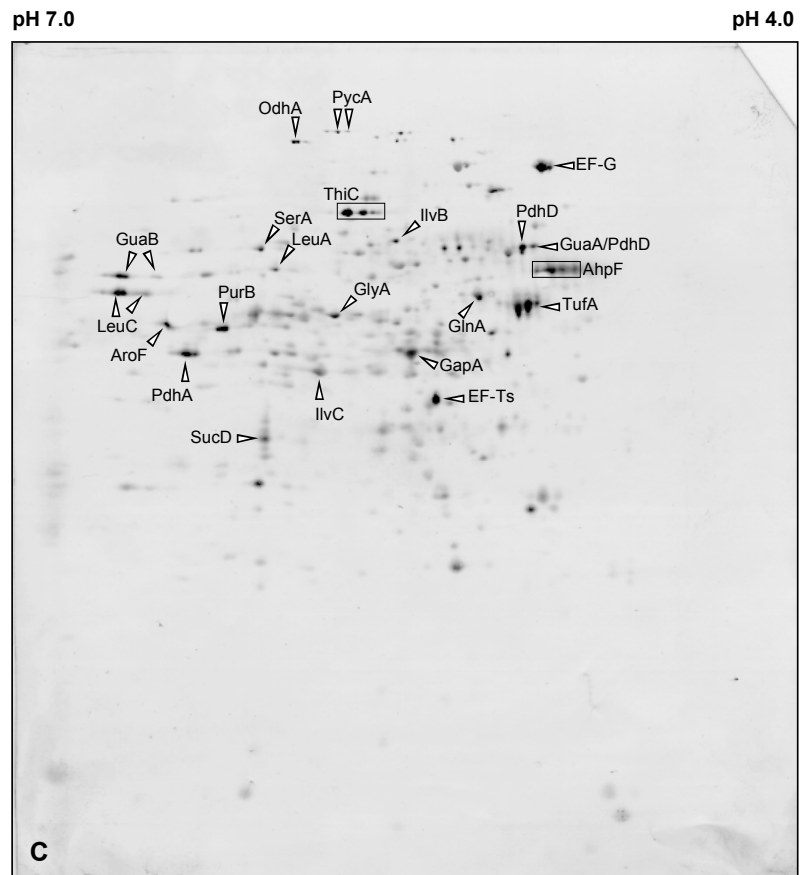
Fig. 8A-D (Contd.)

Screening for Mutants of σ^B-Dependent Genes with Increased Sensitivity against Hydrogen Peroxide in *Bacillus subtilis*

Summary. The alternative sigma factor σ^B of *Bacillus subtilis* controls a large general stress regulon that is induced after the imposition of physical stress or under conditions of energy limitation. After glucose starvation, the general stress response mediates protection against hydrogen peroxide (H_2O_2). Here we demonstrate by means of growth monitoring and survival analysis that *B. subtilis* cells, stimulated by transient heat shock, acquire increased resistance to moderate concentrations of H_2O_2. For *sigB* mutant cells, resistance was observed to be significantly lower. These results indicate that the general stress response contributes to cross-protection against oxidative stress after stimulation by mild or moderate heat shock. In order to identify general stress proteins that are involved in the defense against H_2O_2, of approximative 150 σ^B-dependent genes, 80 mutant strains, cross-protected by heat shock, were analyzed for phenotypical alterations after oxidative stress. A strain with mutation of the *dps*-gene encoding the non-specific DNA binding protein Dps was found to resemble the phenotype of a *sigB* mutant strain. The presented results strengthen the proposed essential antioxidant function of the Dps/PexB protein in *B. subtilis*.

Introduction

In order to cope with a diversity of environmental fluctuations in their natural habitats, bacteria developed highly complex adaptive response networks during their evolution. In the soil-living bacterium *Bacillus subtilis*, the general stress response represents a comprehensive strategy that is activated by the imposition of various stressful conditions in order to confer the cells multiple stress resistance (Hecker & Völker, 2001; Price, 2002). Induction of the general stress regulon, mediated by the alternative sigma factor σ^B,

occurs in response to heat shock, salt, ethanol or acid stress or to starvation of glucose, oxygen or phosphate (Hecker et al., 1996). The activity of σ^B itself is modulated by the action of regulatory 2C-type protein phosphatases that each enforce the formation of a complex between antisigma factor RsbW and its antagonist RsbV (Price, 2000). In the course of partner-switch, σ^B is deliberated from the inhibitory complex with RsbW and is able to effectively compete with vegetative sigma factor σ^A for binding to the RNA polymerase core enzyme (Benson &

Haldenwang, 1992; Benson & Haldenwang, 1993b; Dufour & Haldenwang, 1994; Völker et al., 1994). Altogether, about 150 genes are under the control of σ[B] (Petersohn et al., 1999b; Petersohn et al., 1999c; Petersohn et al., 2001; Price et al., 2001). Besides a large number of proteins with unknown function, proteins homologous to detoxifying catalases, DNA protecting proteins and several dehydrogenases have been identified (Hecker & Völker, 1998).

In contrast to the wealth of information about σ[B]-regulation, the function of the general stress response remained obscure for a long time. The first phenotypical difference between wild-type and *sigB* mutant cells was identified for glucose starved cells challenged with hydrogen peroxide (H_2O_2). While non-growing wild-type cells acquire a nearly complete resistance to otherwise lethal H_2O_2 concentrations (Dowds et al., 1987), non-growing *sigB* mutant cells partially fail to enhance their resistance under these conditions (Engelmann & Hecker, 1996). Furthermore, *sigB* mutant cells were found to be more sensitive to the radical producing agent cumene hydroperoxide compared to wild-type cells (Antelmann et al., 1996). In the following, it was demonstrated that in addition to oxidative stress resistance, σ[B] mediates protection e.g. against severe heat shock and desiccation stress as well as it provides

resistance to the antibiotic rifampin (Völker et al., 1999; Bandow et al., 2002).

Reactive oxygen species (ROS), generated primarily as by-products of normal respiratory activity in aerobic environments, can damage all biologically relevant macromolecules, i.e. nucleic acids, membrane lipids and proteins. Aerobically growing bacteria are able to activate cellular defense and protection systems in order to prevent toxic effects of ROS, especially when amounts of ROS are increased and cells encounter oxidative stress (Farr & Kogoma, 1991; Imlay, 2002). In growing *B. subtilis* cells, the primary defense against increased levels of ROS is the synthesis of a H_2O_2 detoxifying catalase and an alkyl hydroperoxide reductase, DNA protecting proteins and haem biosynthesis proteins (Helmann et al., 2003). The according genes are controlled by the peroxide specific regulator protein PerR and are in part strongly induced after H_2O_2 treatment (Chen et al., 1995; Bsat et al., 1998). When the synthesis of new proteins is unfavored, e.g. under conditions of glucose starvation, the σ[B]-dependent general stress response is suggested to complement peroxide-specific stress proteins in providing antioxidant functions (Engelmann & Hecker, 1996; Hecker & Völker, 2001). Detailed investigation of the σ[B]-dependent catalase KatE and the DNA binding protein Dps revealed that Dps but

not KatE is involved in the resistance to H_2O_2 under the tested conditions (Engelmann & Hecker, 1996; Antelmann et al., 1997b).

Recently, an approach to analyze mutant strains of σ[B]-dependent genes after heat shock, cold shock and ethanol stress was started in order to classify general stress proteins into functional groups (D. Höper & M. Hecker, in preparation). Considering the large number of general stress proteins and the important role of σ[B] in survival after oxidative stress, we suggested that general stress proteins could be involved in a diversity of antioxidant functions. Therefore, in addition to heat shock, cold shock and ethanol stress, a detailed investigation of the putative contribution of general stress proteins to resistance to oxidative stress became likewise necessary.

In the present study, *B. subtilis* wild-type and *sigB* mutant cells were analyzed by means of growth monitoring for the resistance to H_2O_2 after cross-protection by mild or moderate heat treatment, i.e. 48°C or 50°C. To identify general stress proteins involved in H_2O_2 resistance, growth of cross-protected mutant strains was monitored after H_2O_2 challenge and then compared to the phenotype of the *sigB* mutant strain. In order to prove survival rates, cross-protected wild-type cells, *sigB* mutant cells and a *dps* mutant strain were

analyzed for their survival after oxidative stress.

Methods

Bacterial strains and growth conditions. The *B. subtilis* wild-type strain 168 (*trpC2*) (Anagnostopoulos & Spizizen, 1961), its isogenic *sigB* mutant ML6 (*trpC2 sigB*:: ΔHindIII-EcoRV:: *cat*)(Igo et al., 1987) and mutant strains containing mutations in σ[B]-dependent genes (Table 1) were grown aerobically at 37°C in a synthetic medium (Stülke et al., 1993). When appropriate, the medium was supplemented with chloramphenicol (final concentration 5 μg/ml) or erythromycin (5 μg/ml) and lincomycin (25 μg/ml).

For stress experiments, cells were grown exponentially to an OD_{500} value of 0.4 and transferred to 48°C or 50°C for 15 min. Cells were then retransferred back to 37°C, and H_2O_2 was added immediately to the final concentration desired. To limit H_2O_2 effects to a specific time period, exogenous catalase (10 μg/ml, Sigma) was added to the cultures 15 minutes after H_2O_2 addition. To identify phenotypic alterations in H_2O_2 resistance, cell growth was monitored. For significance, all mutants were tested twice. In case of deviation, a third trial of stress experiment was carried out.

For testing cell viability of cross-protected cells after exposure to H_2O_2, cells
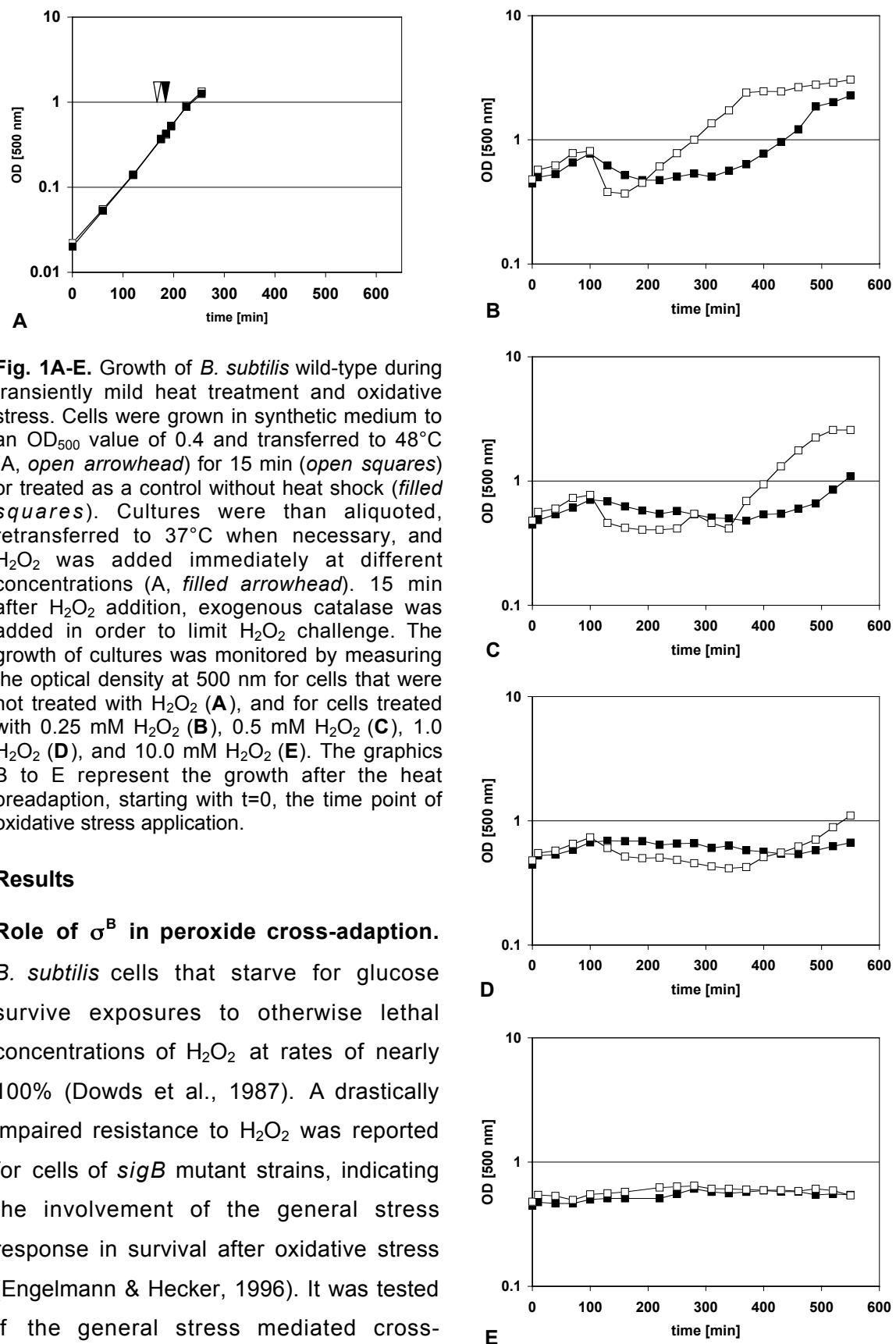
were grown to an OD$_{500}$ value of 0.4 and exposed to 48°C for 15 minutes with subsequent H$_2$O$_2$ challenge as described before. Aliquots of the culture were withdrawn 30 min after stress exposure, and appropriate dilutions were plated on LB agar and incubated overnight.

Table 1: Bacterial strains.

Strain	Relevant characteristics	Reference
BFS 205	*trpC2 ywmE::pEC6 Em^r*	(Kobayashi et al., 2003)
BFS 230	*trpC2 ywlB::pEC35 Em^r*	(Kobayashi et al., 2003)
BFS 410	*trpC2 yraA::pM2yraA1 Em^r*	(Kobayashi et al., 2003)
BFS 601	*trpC2 ytaB::pEU1 Em^r*	(Kobayashi et al., 2003)
BFS 620	*trpC2 ytiA::pEU20 Em^r*	(Kobayashi et al., 2003)
BFS 625	*trpC2 dps::pEU25 Em^r*	(Kobayashi et al., 2003)
BFS 675	*trpC2 yocK::pEU75 Em^r*	(Kobayashi et al., 2003)
BFS 684	*trpC2 yocB::pEU84 Em^r*	(Kobayashi et al., 2003)
BFS 834	*trpC2 yjgB::pjgB Em^r*	(Kobayashi et al., 2003)
BFS 836	*trpC2 yjgC::pjgC Em^r*	(Kobayashi et al., 2003)
BFS 860	*trpC2 yjgD::pjgD Em^r*	(Kobayashi et al., 2003)
BFS 1121	*trpC2 rnr::pAC2 Em^r*	(Kobayashi et al., 2003)
BFS 1239	*trpC2 ywtG::pywtG Em^r*	(Kobayashi et al., 2003)
BFS 1316	*trpC2 ywsB::pmutin4::ywsB Em^r*	(Kobayashi et al., 2003)
BFS 1412	*trpC2 yugU::pKTH3436 Em^r*	(Kobayashi et al., 2003)
BFS 1659	*trpC2 yhdN::pYHDN1 Em^r*	(Kobayashi et al., 2003)
BFS 1702	*trpC2 yhxD::pYHXD1 Em^r*	(Kobayashi et al., 2003)
BFS 1818	*trpC2 ykzA::pDU1818 Em^r*	(Kobayashi et al., 2003)
BFS 1819	*trpC2 guaD::pDU1819 Em^r*	(Kobayashi et al., 2003)
BFS 1820	*trpC2 ykgA::pDU1820 Em^r*	(Kobayashi et al., 2003)
BFS 2043	*trpC2 yoxC::pM2043 Em^r*	(Kobayashi et al., 2003)
BFS 2438	*trpC2 ysnF::pYSNFK Em^r*	(Kobayashi et al., 2003)
BFS 2477	*trpC2 yvrE::pYVREK Em^r*	(Kobayashi et al., 2003)
BFS 2490	*trpC2 yvgO::pYVGOK Em^r*	(Kobayashi et al., 2003)
BFS 2491	*trpC2 yvgN::pYVGNK Em^r*	(Kobayashi et al., 2003)
BFS 2629	*trpC2 ylxP::plxP Em^r*	(Kobayashi et al., 2003)
BFS 2673	*trpC2 ymzB::pMUTIN4/ymzB1B2 Em^r*	(Kobayashi et al., 2003)
BFS 2841	*trpC2 yjbC::pSG1829 Em^r*	(Kobayashi et al., 2003)
BFS 2842	*trpC2 yjbD::pSG1830 Em^r*	(Kobayashi et al., 2003)
BFS 4002	*trpC2 yxlJ::pMutin1 Em^r*	(Kobayashi et al., 2003)
BFS 4003	*trpC2 katX::pMutin1 Em^r*	(Kobayashi et al., 2003)
BFS 4012	*trpC2 yxkO::pMutin1 Em^r*	(Kobayashi et al., 2003)
BFS 4043	*trpC2 yxiS::pMutin2 Em^r*	(Kobayashi et al., 2003)
BFS 4044	*trpC2 katE::pMutin2 Em^r*	(Kobayashi et al., 2003)
BFS 4101	*trpC2 csbC::pMutin1 Em^r*	(Kobayashi et al., 2003)
BFS 4117	*trpC2 yxnA::pMutin2 Em^r*	(Kobayashi et al., 2003)
BFS 4120	*trpC2 yxaB::pMutin2 Em^r*	(Kobayashi et al., 2003)

Table 1: Bacterial strains cont.

BFS 4145	*trpC2 yycD::pMutinT3 Em^r*	(Kobayashi et al., 2003)
BFS 4195	*trpC2 yaaH::pMutin1 Em^r*	(Kobayashi et al., 2003)
BFS 4196	*trpC2 yaaI::pMutinT3 Em^r*	(Kobayashi et al., 2003)
BFS 4227	*trpC2 yacL::pMutinT3 Em^r*	(Kobayashi et al., 2003)
BFS 4281	*trpC2 cypC::pMutin2 Em^r*	(Kobayashi et al., 2003)
BFS 4282	*trpC2 ybyB::pMutin1 Em^r*	(Kobayashi et al., 2003)
BFS 4225	*trpC2 radA::pMutinT3 Em^r*	(Kobayashi et al., 2003)
BFS 4226	*trpC2 yacK::pMutinT3 Em^r*	(Kobayashi et al., 2003)
BFS 4316	*trpC2 ycbK::pMutin2 Em^r*	(Kobayashi et al., 2003)
BFS 4321	*trpC2 ycbP:: Em^r*	(Kobayashi et al., 2003)
BFS 4337	*trpC2 ycdF::pMutin2 Em^r*	(Kobayashi et al., 2003)
BFS 4341	*trpC2 yceD::pMutin2 Em^r*	(Kobayashi et al., 2003)
BFS 4342	*trpC2 yceE::pMutin2 Em^r*	(Kobayashi et al., 2003)
BFS 4343	*trpC2 yceF::pMutin2 Em^r*	(Kobayashi et al., 2003)
BFS 4344	*trpC2 yceG:: Em^r*	(Kobayashi et al., 2003)
BFS 4401	*trpC2 gabD::pMutin2 Em^r*	(Kobayashi et al., 2003)
BFS 4423	*trpC2 ydaD::pMutinT3 Em^r*	(Kobayashi et al., 2003)
BFS 4424	*trpC2 ydaE::pMutinT3 Em^r*	(Kobayashi et al., 2003)
BFS 4426	*trpC2 ydaG::pMutinT3 Em^r*	(Kobayashi et al., 2003)
BFS 4435	*trpC2 ydaP::pMutinT3 Em^r*	(Kobayashi et al., 2003)
BFS 4437	*trpC2 ydaS::pMutinT3 Em^r*	(Kobayashi et al., 2003)
BFS 4438	*trpC2 ydaT::pMutinT3 Em^r*	(Kobayashi et al., 2003)
BFS 4442	*trpC2 ydbD::pMutinT3 Em^r*	(Kobayashi et al., 2003)
BFS 4568	*trpC2 yfnI::pMutin2 Em^r*	(Kobayashi et al., 2003)
BFS 4597	*trpC2 yflT::pMutin2 Em^r*	(Kobayashi et al., 2003)
BFS 4610	*trpC2 yflH::pMutin2 Em^r*	(Kobayashi et al., 2003)
BFS 4617	*trpC2 yflA::pMutin2 Em^r*	(Kobayashi et al., 2003)
BFS 4627	*trpC2 yfkM::pMutin2 Em^r*	(Kobayashi et al., 2003)
BFS 4630	*trpC2 yfkJ::pMutin2 Em^r*	(Kobayashi et al., 2003)
BFS 4631	*trpC2 yfkI::pMutin2 Em^r*	(Kobayashi et al., 2003)
BFS 4632	*trpC2 yfkH::pMutin2 Em^r*	(Kobayashi et al., 2003)
BFS 4690	*trpC2 yfhD::pMutin2 Em^r*	(Kobayashi et al., 2003)
BFS 4691	*trpC2 yfhE::pMutin2 Em^r*	(Kobayashi et al., 2003)
BFS 4692	*trpC2 yfhF::pMutin2 Em^r*	(Kobayashi et al., 2003)
BFS 4697	*trpC2 yfhK::pMutin2 Em^r*	(Kobayashi et al., 2003)
BFS 4698	*trpC2 yfhL::pMutin2 Em^r*	(Kobayashi et al., 2003)
BFS 4699	*trpC2 yfhM::pMutin2 Em^r*	(Kobayashi et al., 2003)
BFS 4700	*trpC2 csbB::pMutin2 Em^r*	(Kobayashi et al., 2003)
BFS 4728	*trpC2 yqjL::pMutinT3 Em^r*	(Kobayashi et al., 2003)
BFS 4770	*trpC2 yqxL::pMutinT3 Em^r*	(Kobayashi et al., 2003)
BFS 4771	*trpC2 yqhB::pMutinT3 Em^r*	(Kobayashi et al., 2003)
BFS 4773	*trpC2 yqgZ::pMutinT3 Em^r*	(Kobayashi et al., 2003)
BFS 4792	*trpC2 sodA:: Em^r*	(Kobayashi et al., 2003)

A

B

Fig. 1A-E. Growth of *B. subtilis* wild-type during transiently mild heat treatment and oxidative stress. Cells were grown in synthetic medium to an OD_{500} value of 0.4 and transferred to 48°C (A, *open arrowhead*) for 15 min (*open squares*) or treated as a control without heat shock (*filled squares*). Cultures were than aliquoted, retransferred to 37°C when necessary, and H_2O_2 was added immediately at different concentrations (A, *filled arrowhead*). 15 min after H_2O_2 addition, exogenous catalase was added in order to limit H_2O_2 challenge. The growth of cultures was monitored by measuring the optical density at 500 nm for cells that were not treated with H_2O_2 (**A**), and for cells treated with 0.25 mM H_2O_2 (**B**), 0.5 mM H_2O_2 (**C**), 1.0 H_2O_2 (**D**), and 10.0 mM H_2O_2 (**E**). The graphics B to E represent the growth after the heat preadaption, starting with t=0, the time point of oxidative stress application.

C

D

E

Results

Role of σ^B in peroxide cross-adaption.

B. subtilis cells that starve for glucose survive exposures to otherwise lethal concentrations of H_2O_2 at rates of nearly 100% (Dowds et al., 1987). A drastically impaired resistance to H_2O_2 was reported for cells of *sigB* mutant strains, indicating the involvement of the general stress response in survival after oxidative stress (Engelmann & Hecker, 1996). It was tested if the general stress mediated cross-

protection against H_2O_2 is also provided after a heat stress. Therefore, *B. subtilis* cells were exposed transiently to 48°C and subsequently challenged with different concentrations of H_2O_2 (Fig. 1). To limit stress exposure, 15 minutes after H_2O_2 addition, exogenous catalase was added to the cultures and cell growth was monitored. *B. subtilis* cells treated with mild heat stress did not reveal any impairments of growth, when compared to exponentially growing cells (Fig. 1A). When cells were pretreated with temperature upshift, they regained growth significantly earlier than unprotected, exponentially growing cells after the cultures were challenged with differing concentrations of H_2O_2 (Fig. 1B-E). In fact, for all H_2O_2 concentrations tested except 10 mM H_2O_2, cross-protected cultures showed a significantly shorter period of recovery, indicating an increase in resistance to oxidative stress mediated by heat-stress-protection.

Since the general stress response is strikingly induced after heat stress (Hecker et al., 1996), we asked, if the observed cross-protection involves the action of general stress proteins. Therefore, wild-type and Δ*sigB* *B. subtilis* cells were exposed transiently to 48°C and subsequently challenged with different concentrations of H_2O_2 (Fig. 2). Wild-type and Δ*sigB* mutant cells showed no difference in growth after exposure to a mild heat shock without exposure to oxidative stress. When cultures were treated with H_2O_2 after cross-protection by mild heat stress, comparison of wild-type and *sigB* mutant growth revealed that all H_2O_2 concentrations tested had stronger effects on growth behavior of *sigB* mutant cells. The most significant difference in time of recovery between wild-type and Δ*sigB* cells was observed for 0.25 mM H_2O_2. Thus, the strongest effect of the general stress response was observed in the presence of the lowest H_2O_2 concentration tested. In contrast, the difference of growth recovery was only marginal when cultures were challenged with 10 mM H_2O_2. These results indicated clearly that cross-protection after mild heat treatment is mediated by the general stress response. Interestingly, cultures pretreated with 4% ethanol instead of heat cross-protection showed similar effects in the time of recovery between wild-type and *sigB* mutant cells when 0.25 mM H_2O_2 was applied (data not shown). Even without a preadaptation stimulus the period of replication stop after 0.25 mM H_2O_2 was extended in Δ*sigB* cells compared to the wild-type cells. This indicates involvement of the general stress response mediated by direct activation of σ^B after oxidative stress (data not shown).

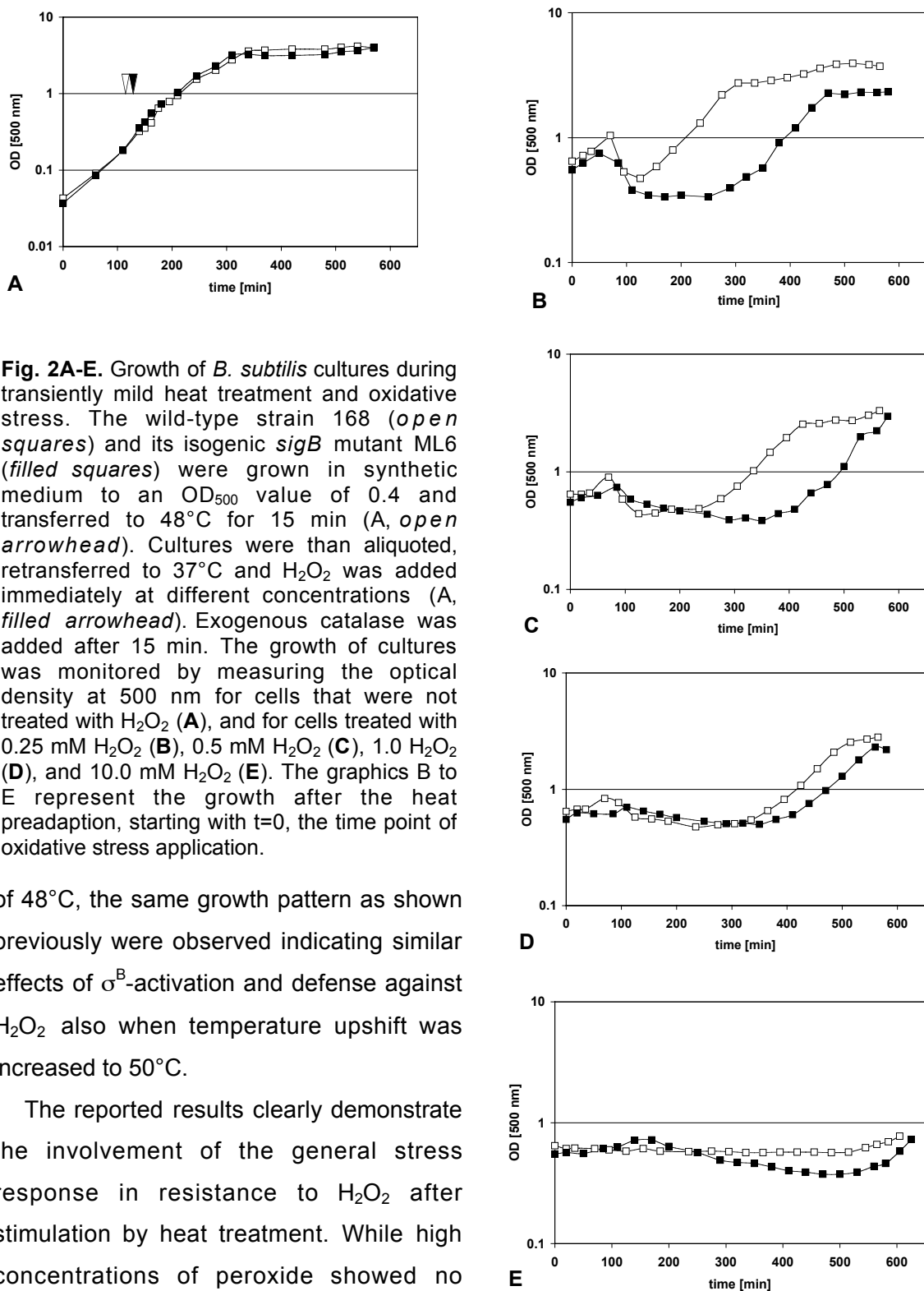When cross-protection was stimulated by transient heat stress using 50°C instead

Fig. 2A-E. Growth of *B. subtilis* cultures during transiently mild heat treatment and oxidative stress. The wild-type strain 168 (*open squares*) and its isogenic *sigB* mutant ML6 (*filled squares*) were grown in synthetic medium to an OD_{500} value of 0.4 and transferred to 48°C for 15 min (A, *open arrowhead*). Cultures were than aliquoted, retransferred to 37°C and H_2O_2 was added immediately at different concentrations (A, *filled arrowhead*). Exogenous catalase was added after 15 min. The growth of cultures was monitored by measuring the optical density at 500 nm for cells that were not treated with H_2O_2 (**A**), and for cells treated with 0.25 mM H_2O_2 (**B**), 0.5 mM H_2O_2 (**C**), 1.0 H_2O_2 (**D**), and 10.0 mM H_2O_2 (**E**). The graphics B to E represent the growth after the heat preadaption, starting with t=0, the time point of oxidative stress application.

of 48°C, the same growth pattern as shown previously were observed indicating similar effects of σ^B-activation and defense against H_2O_2 also when temperature upshift was increased to 50°C.

The reported results clearly demonstrate the involvement of the general stress response in resistance to H_2O_2 after stimulation by heat treatment. While high concentrations of peroxide showed no differences, for moderate concentrations of H_2O_2 significant differences in the period of recovery between wild-type and *sigB* mutant cells were observed.

Mutant screening. From about 150 genes controlled by σ^B, 80 strains with mutations in genes of interest were available (Kobayashi et al., 2003). To test, whether the according proteins are involved in peroxide resistance or not, a screening of mutant strains was carried out. Because of the fact that the observed growth recovery showed most significant differences with 0.25 mM H_2O_2 between wild-type and *sigB* mutant cells, this concentration was chosen. Therefore, strains with mutations in σ^B-dependent genes were exposed to heat treatment with 50°C for 15 min and than retransferred back to 37°C. Immediately, H_2O_2 was added to the culture to the final concentration of 0.25 mM H_2O_2. Exogenous catalase was added after 15 minutes of H_2O_2 challenge to limit stress exposure and cell growth was monitored. The obtained growth curves of the mutant strains were overlaid in one diagram together with representative growth curves of the wild-type strain 168 and the *sigB* mutant (Fig. 3). In table 1 all mutant strains that were tested within the screening process are listed. Examining the growth curves with special emphasis of the OD values, it can be observed that the time of recovery is the shortest for *B. subtilis* wild-type cells. The predominate number of mutant strains is represented by the majority of growth curves and resumed growth shortly after *B. subtilis* wild-type cells. For the *sigB*

mutant, a delay of about 100 min and more was detected before the cells resumed growth and reached an OD value of 1.0. Cells of the mutant strain BFS 625 resembled phenotypical characteristics of the *sigB* mutant concerning the delay in recovery of growth. *B. subtilis* BFS 625 carries a mutation in the gene *dps* encoding the DNA binding protein Dps. Thus, σ^B-dependent Dps represents a general stress protein that has been identified to be involved in resistance to oxidative stress after induction of the general stress response by means of moderate heat treatment.

Influence of σ^B on survival after oxidative stress. Exponentially growing cultures of *B. subtilis* cells that are challenged with H_2O_2 showed increased rates of cell death (Dowds et al., 1987; Murphy et al., 1987). When *B. subtilis* cells are cross-protected by means of glucose starvation, the killing rate decreased. In contrast, when glucose starved *sigB* mutant cells were tested for survival after H_2O_2 challenge, the cells partly failed to show increased survival rates (Engelmann & Hecker, 1996).

It was tested if the observed differences in the time of recovery of wild-type and *sigB* mutant cells after cross-protection by mild heat treatment are an indication of different rates of cell death. Therefore, survival
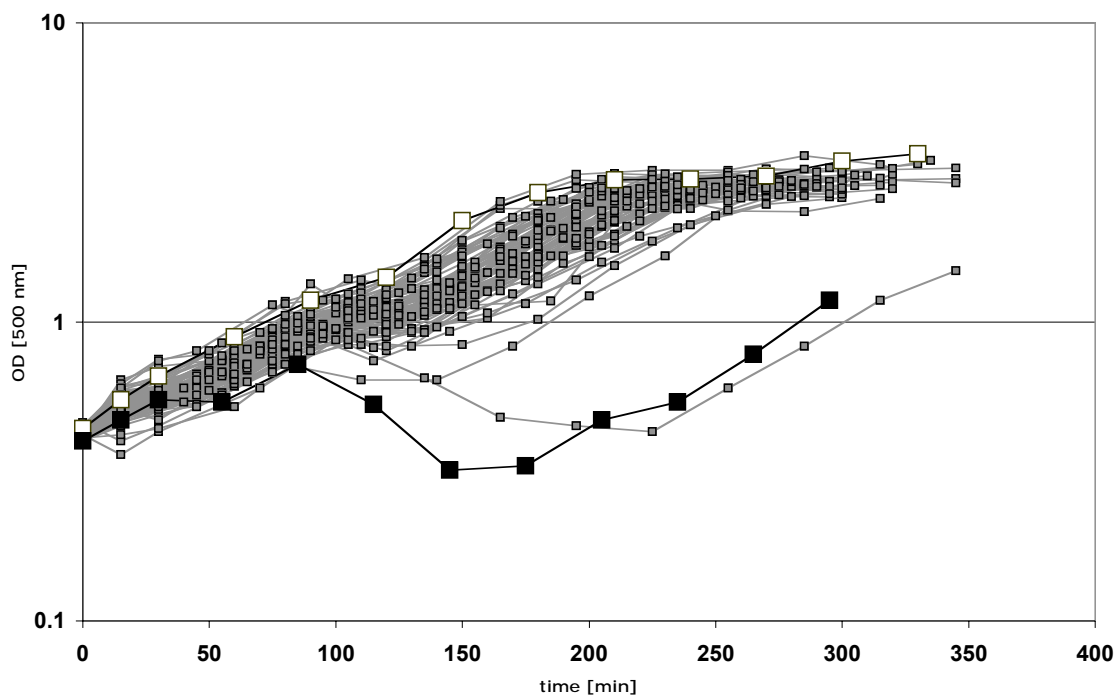
Fig. 3. Growth of cross-protected *B. subtilis* mutant strains (*gray squares*) in comparison to wild-type (*open squares*) and *sigB* mutant cells (*black squares*). Cells were challenged with 0.25 mM H_2O_2 after cultures were preadapted by a transient heat shock of 50°C. After 15 min of H_2O_2 challenge, exogenous catalase was added. Growth is monitored with the beginning of heat stress. The mutant strain with significant delay in growth recovery represents BFS 625.

analysis were carried out. Consequently, exponentially growing cells were exposed to transient heat shock with 48°C and challenged with different concentrations of H_2O_2. Samples were drawn after 30 min and survival was analyzed by plating aliquotes. Compared to a control that was not treated with H_2O_2, all oxidatively stressed cultures showed increased rates of cell death (Fig. 4). For concentrations of 0.25, 0.5 and 1.0 mM H_2O_2 the rate of survival was higher for wild-type cells compared to *sigB* mutant cells. The strongest difference in survival between wild-type and *sigB* mutant cells was observed after challenging the cultures with

0.5 or 1.0 mM H_2O_2. Under the tested conditions, no difference in the rate of survival was detected for cells treated with 10.0 mM H_2O_2. Similar results were obtained from samples that were withdrawn after 60 min of oxidative stress exposure (data not shown). The observed results indicate that the rate of killing is higher in *sigB* mutant cells compared to wild-type cells after heat cross-adaptation as this was expected from growth analysis. While *B. subtilis* wild-type cells survive concentrations of 0.25, 0.5 and 1.0 H_2O_2 from 10% to 1%, *sigB* mutant cells display survival rates of 1% to 0.01% or even as low as 0.001%. In contrast to the strongest

difference in the growth recovery experiments (0.25 mM), the maximum difference in the rate of cell death between wild-type and *sigB* mutant cells was detected when concentrations of 0.5 mM or 1.0 mM H_2O_2 were used. The results obtained demonstrate that the general stress response is essentially involved in the protection of survival after oxidative stress after cross-protection by mild heat stress.

It was tested, if Dps contributes to survival when cross-protected by mild heat shock. For this reason, the mutant strain BFS 625 was analyzed for survival rates after H_2O_2 challenge and compared to wild-type and *sigB* mutant cells (Fig. 5). Survival rates of BFS 625 resembled survival rates of *sigB* mutant cells, indicating that the general stress protein Dps is essential for survival after cross-protection by mild heat shock.

Discussion

Sigma factor σ^B of *B. subtilis* was the first alternative sigma factor that was identified (Haldenwang & Losick, 1979; Igo et al., 1987). In contrast to the wealth of information about regulation of σ^B-activity and to the growing list of genes under the control of σ^B, its physiological function remained not well understood for a long time (Hecker et al., 1996). The first indication for a role of the postulated
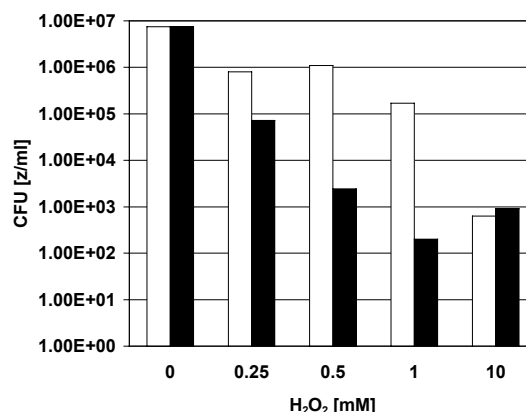
Fig. 4. Survival rates of *B. subtilis* wild-type cells (*white bars*) and *sigB* mutant cells (*black bars*). Cells were cross-protected by means of heat shock (48°C) and subsequently challenged with H_2O_2 concentrations as indicated.
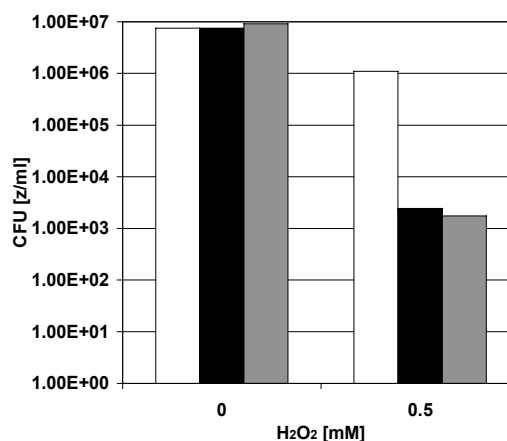

Fig. 5. Survival rates of *B.subtilis* BFS 625 (*gray bars*) in comparison to wild-type (*white bars*) and *sigB* mutant (*black bars*) cells. Cross-protection was achieved by heat shock (48°C), oxidative stress was applied by addition of 0.5 mM H_2O_2.

general stress regulon emerged when *sigB* mutant cells were found to be highly sensitive to H_2O_2 or to cumene hydroperoxide (Antelmann et al., 1996; Engelmann & Hecker, 1996). Recently, it was demonstrated that the σ^B-regulon is not only involved in adaptation to oxidative stress but is also induced by H_2O_2, although

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only weakly (Helmann et al., 2003). Indications for σ^B-activation after exposure to H_2O_2 were also observed by means of transcriptome and proteome analysis in this thesis. However, the weak induction of the general stress response observed after treatment with protective concentrations of H_2O_2 (58 µM) (Dowds, 1994) might be of minor physiological importance in the absence of additional stimuli.

Under conditions of glucose starvation, the general stress response, induced at the onset of the stationary phase, provides non-specific resistance to oxidative stress (Engelmann & Hecker, 1996). Our data clearly demonstrate that resistance to ROS is acquired by a transient heat shock. The absence of σ^B in a *sigB* mutant strain results in the decrease of the otherwise observed resistance. Thus, cross-protection against H_2O_2 by σ^B is not limited to conditions of energy depletion but is also provided after physical stress, i.e. transient heat shock. Since heat stress is suggested to be a typical stimulus in the upper layers of soil, acquiring increased resistance to ROS may provide a survival advantage for *B. subtilis* cells to face concomitant or future oxidative stress. Our results are in accordance with the postulated concept for the general stress response that is to confer *B. subtilis* cells with a comprehensive multiple stress resistance for defeating future stress (Hecker & Völker,

2001).

Detailed inspection of the growing list of general stress proteins revealed the putative involvement of proteins homologous to detoxifying catalases, DNA protecting proteins and several dehydrogenases (Hecker & Völker, 1998; Hecker & Völker, 2001). The results we presented here support earlier reports of our group that not catalase KatE but Dps is essential for conferring non-specific antioxidant function (Engelmann & Hecker, 1996; Antelmann et al., 1997a). Under the conditions tested, no other mutant strain than BFS 625 of a σ^B-dependent general stress gene showed significantly increased sensitivity to H_2O_2. For example, strain BFS 4044, representing the *katE* mutant, displayed no impairments of growth in our analysis. With respect to the numerous biological targets of reactive oxygen species, the identification of Dps as the sole protein involved in general stress mediated protection against ROS appears to be astonishing at first. On the other hand, the mechanism whereby H_2O_2 is cytotoxic to bacterial cells was demonstrated to depend on the H_2O_2 concentration used. For *Escherichia coli*, it was reported that concentrations between 1–2 mM H_2O_2 may cause killing by transition metal-mediated DNA damage (Imlay & Linn, 1986; Dowds, 1994). At present, we do not know which mode of

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killing is responsible for the observed phenotypes in *B. subtilis*. The finding that Dps plays a dominant role in resistance to H_2O_2 after cross-adaptation suggests a severe damage of DNA when Dps is absent. In *E. coli*, a *dps* mutant was found to be highly sensitive to the oxidative agent hypochlorus acid (HOCl) in the stationary phase, but no protective effect was observed for RpoS-dependent catalase single mutants (Dukan & Touati, 1996). In the genome of *B. subtilis*, two Dps/PexB homologues have been found. The gene encoding MrgA is under control of the peroxide specific regulator PerR and is one of the most prominent induced genes in response to H_2O_2 and paraquat treatment. Altogether, these findings suggest a major protective function in non-specific DNA protection after oxidative stress in *B. subtilis*.

Our observations do not exclude the involvement of other general stress proteins, especially under conditions of nutrient limitation. In an approach to identify genes of the anaerobic bacterium *Clostridium perfringens* that are involved in the adaptive response to oxidative stress, two proteins homologous to the gene products of *ydaD* and *ycdF* of *B. subtilis* were found (Briolat & Reysset, 2002). Mutation of the genes *ydaD* or *ycdF* in *C. perfringens* resulted in a lowered resistance to H_2O_2, especially when cells were in a non-growing state. In *B. subtilis*, both proteins are under control of σ^B, but they were not identified to be involved in oxidative stress resistance after cross-protection by heat shock followed by H_2O_2 challenge.

The investigation of a large set of mutants in σ^B-dependent general stress genes enabled a comprehensive view of phenotypic changes after oxidative stress in order to identify the major players. We have shown here that the non-specific DNA-binding protein Dps plays an essential role in resistance of oxidative stress after cross-protecting cells by transient heat stress. Thus, the presented work indicates a highly important role for Dps in maintaining DNA integrity after oxidative stress.

Functional Analysis of Thioredoxin in *Bacillus subtilis*

Summary. The thiol-disulfide oxidoreductase thioredoxin is an essential gene in *Bacillus subtilis*. In *Escherichia coli,* thioredoxin is involved in the re-reduction of disulfide bonds in reductive enzymes, e.g. 3'-phosphoadenosine 5'-phosphosulfate reductase and in maintaining the thiol state of cysteine-containing proteins in the cell. In this work, a *trxA* conditional mutant was analyzed by means of growth monitoring and proteomics. When cells were depleted of thioredoxin, a transient reduction of growth was observed. Within this period, the cells responded by induction of several proteins including proteins involved in sulfur-assimilation. Replacing sulfate by alternative sulfur-sources did not result in a reduction of growth in the absence of TrxA. Furthermore, only minor changes on the level of protein synthesis were observed, when sulfate was exchanged by methionine or thiosulfate. Our results indicate that *B. subtilis* TrxA is primarily essential for the reactivation of 3'-phosphoadenosine 5'-phosphosulfate reductase CysH.

Introduction

Cysteine residues play important roles in protein function, e.g. in stabilizing protein structure or providing sites for easily reversible oxidation/reduction chemistry. In extracytoplasmic proteins cysteines are primarily involved in the generation of disulfide bonds which provide structural stability in protein folding (Bardwell, 1994; Rietsch & Beckwith, 1998). For intracellular proteins, it is suggested that in the reducing environment of the cytoplasm cysteine residues are predominantly maintained in their reduced form. The thiol functional group of cysteines in intracellular proteins is often involved in metal binding or enzymic redox chemistry. However, transient disulfide bond formation is also found in cytoplasmic reductive enzymes as part of their catalytic cycle, e.g. in ribonucleotide reductase, 3'-phosphoadenosine 5'-phosphosulfate (PAPS) reductase and methionine sulfoxide reductase (Holmgren, 1989). Under the condition of increased amount of reactive oxygen species, non-native disulfide bonds are suggested to be generated also in otherwise thiol-containing cytoplasmic proteins as a consequence of perturbation of the intracellular redox balance, e.g. in OxyR of *Escherichia coli* (Aslund & Beckwith, 1999a).

To maintain the thiol state of intracellular proteins or to generate properly placed disulfide bonds in extracytoplasmic proteins, cellular systems are provided with thiol-disulfide oxidoreductases of the thioredoxin superfamily (Aslund & Beckwith, 1999b). In *E. coli* for example, thioredoxin 1, 2 and 3 as well as glutaredoxin 1, 2 and

3 were identified to re-reduce disulfides of reductive enzymes or to maintain the thiol state of native cysteine thiols. In addition to the overlap of function that has been observed among these proteins, evidence was found that each protein fulfills also a specific role in *E. coli* (for short review see Aslund & Beckwith, 1999b).

In contrast to the diverse sets of thioredoxins and glutaredoxins in *E. coli*, only a single intracellular system was identified in *Bacillus subtilis* up till now (Scharf et al., 1998). The thioredoxin system, composed of thioredoxin TrxA and NADPH-dependent thioredoxin reductase TrxB, is suggested to maintain the role of a reductive thiol-disulfide oxidoreductase and thioredoxin-related antioxidant functions in the cytoplasm of this bacterium. Investigations of gene regulation revealed that expression of *trxA* gene is strongly enhanced by a variety of stresses including hydrogen peroxide and diamide challenge (Scharf et al., 1998; Leichert et al., 2003). Recently it was demonstrated that *trxA* and *trxB* are subject to Spx dependent regulation in response to disulfide stress (Nakano et al., 2003). As attempts to construct a null mutant in the *trxA* gene failed, and since a strain with an artificially inducible *trxA* gene was demonstrated to stop growth in the absence of its inducer, TrxA was suggested to represent an essential protein in *B. subtilis* (Scharf et al.,

1998). However, in the genome sequence of *B. subtilis*, open reading frames were identified that encode proteins with homologies to TrxA indicating their putative involvement in thiol-disulfide redox chemistry (Kunst et al., 1997).

To identify the physiological function of thioredoxin TrxA in *B. subtilis*, we analyzed the response of a *trxA* conditional mutant depleted of TrxA protein by means of two-dimensional (2D) protein gel electrophoresis. The obtained results led us to the analysis of sulfur-assimilation. The utilization of methionine, thiosulfate and sulfite replacing sulfate as the sole sulfur source under the conditions of TrxA depletion was tested by means of growth monitoring and gene expression pattern which were visualized by 2D protein electrophoresis.

Methods

Growth analysis. The *B. subtilis* wild-type strain 168 (Anagnostopoulos & Spizizen, 1961) and the *trxA* mutant strain IΔ*trxA* (*trpC2 trxA*::pMutin)(Scharf et al. 1998) were cultivated aerobically at 37°C in a synthetic medium (Stülke et al., 1993). To provide TrxA protein in IΔ*trxA* during growth, the inducing agent isopropyl-ß-D-thiogalactopyranoside (IPTG) was added to the medium (final concentration 50 µM). When appropriate, sulfate was replaced as the sole sulfur source. Therefore, all

sulfate-containing supplements of the synthetic medium were replaced by aequimolar amounts of their chloride salts. In addition, an appropriate sulfur source was added to the growth medium. Erythromycine (final concentration 5µg/ml) and lincomycine (25 µg/ml) were added to I∆trxA cultures for selection of trxA::pMutin cells. In order to deplete cultures of TrxA protein and/or to exchange the sulfur source during exponential growth, late exponentially phase cells (OD value of 0.6 to 0.7) were centrifuged (5 min, 8000 rpm, 37°C) and cell pellets were transferred to prewarmed medium and resuspended immediately. Cultivation was then continued as usual.

Northern analysis. Cell harvesting and preparation of total RNA were performed as described by Jürgen et al. (in preparation). The Northern hybridization procedure was carried out as described previously (Eymann *et al.*, 2002). Chemiluminescence was detected with the Lumi-Imager (Roche Diagnostics). The DIG-labeled RNA probes were synthesized by *in vitro* transcription with T7 RNA polymerase and specific PCR products as templates. Synthesis of the templates by PCR was performed using the oligonucleotide pairs TrxA-5' (5'-GCTATCGTAAAAGCAACTG-3') and TrxA-3' (5'-<u>CTAATACGACTCACTATAGG GAGA</u>TCAACTACTTCTCCGTCTT-3') for

trxA specific probe. The underlined sequence indicates the T7 promoter region.

Proteome analysis. Preparation of protein extracts and two dimensional protein gel electrophoresis were carried out as described previously (Büttner et al., 2001). Crude protein extracts containing 100 µg or 500 µg were loaded onto IPG-strips in the pH range 4-7 (Amersham Pharmacia Biotech) for the first dimension of 2D gel electrophoresis. Electrophoretically separated proteins were visualized by staining with silver or colloidal coomassie dye. Data were analyzed by comparison of the 2D protein patterns using Delta2D software (Version 3.1; Decodon).

Results and discussion

TrxA depletion and *trxA* mRNA synthesis. Since *trxA* is suggested to represent an essential gene in *B. subtilis* and cells of the conditional mutant were reported not to be viable without addition of IPTG as inducer for *trxA* transcription (Scharf et al., 1998), growth analysis of I∆trxA was performed (Fig. 1). Addition of 50 µM IPTG to the growth medium enabled cells of I∆trxA to replicate with a rate widely comparable to the growth rate of *B. subtilis* wild-type cells in synthetic medium (60 min respectively 45 min doubling time). When the medium with 50 µM IPTG of exponentially growing cells was exchanged

against medium without IPTG, the growth rate was observed to decrease significantly 90 minutes after the transfer. The impairment of growth indicated that cells were depleted of TrxA protein 90 minutes after replacing IPTG-rich medium against medium without IPTG. Cells which were transferred from a 50 µM IPTG-containing medium to a medium containing 25 µM IPTG showed also a reduction of the doubling time albeit less distinct. The growth behaviour in medium with lower concentrations of IPTG from the beginning of cultivation, led to the suggestion that the mutation is not stable with less than 25 µM IPTG (data not shown). Moreover, the impairment of growth that was observed when IΔtrxA cells were transferred to medium without IPTG was found to be only transient. These results indicated that the mutant harboring the chromosomally integrated trxA::pMutin construct is not very stable under the tested conditions. Interestingly, Pragai & Harwood (2000) obtained similar results for pMutin-based construction of a conditional mutant with another essential gene of B. subtilis. These authors identified the optimized "oid" operator region of the LacI repressor protein in the P_{spac} promoter of the vector to attain single transitions at nucleotide 10 with high frequency when pMutin4 was used for construction of an ysxC conditional mutant. This might be also true for
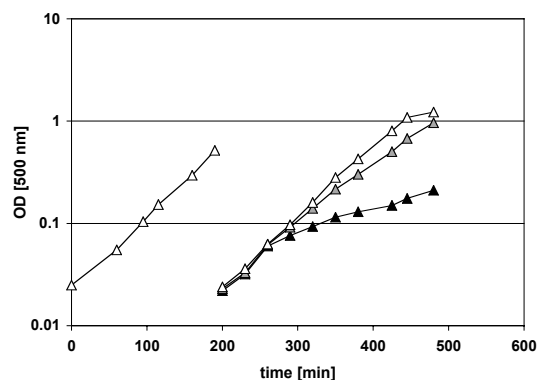

Fig 1. Growth of *B. subtilis* IΔ*trxA* with and without IPTG. Cells were grown exponentially in synthetic medium containing 50 µM IPTG (*open triangles*). When cells reached the late exponential phase, original medium was replaced by medium containing no IPTG (*black triangles*), 25 µM IPTG (*gray triangels*) or 50 µM IPTG (*open triangles*).

B. subtilis IΔ*trxA* carrying *trxA*::pMutin4 integrated into the chromosome.

Northern analysis were performed to test whether the amount of *trxA* mRNA is reduced in consequence to IPTG depletion, or not (Fig. 2). Samples were drawn from exponentially growing cells 45, 75, 135 and 195 minutes after transferring the cells to medium without IPTG. In order to compare *trxA* mRNA levels in the mutant to wild-type levels during exponential growth, mRNA samples from *B. subtilis* 168 were also prepared and analyzed (Fig. 2). During exponential growth in the presence of 50 µM IPTG, *trxA* mRNA is synthesized indicated by the small signal representing the *trxA* mRNA transcript synthesized from the P_{spac}-inducible promoter. In wild-type cells, *trxA* mRNA is also observed and represented by the small signal albeit the amount appears to be slightly lower

compared to the IΔ*trxA* IPTG-dependent transcript. 45 and 75 minutes after depletion of IPTG, the *trxA* gene was observed to be strongly repressed at mRNA level in IΔ*trxA*. 135 and 195 minutes after IPTG depletion, a weak signal indicated reactivation of transcription that might be due to mutation of the operator sequence. Altogether, the data from growth monitoring and northern analysis indicate that within the small time period of about 200 minutes after transferring cells to IPTG-free medium, IΔ*trxA* is depleted of functional TrxA protein.

Change of protein synthesis pattern. In *E. coli* as well as in other organisms, thioredoxin 1 is essential for the reduction of disulfide bonds (Aslund & Beckwith, 1999a; Aslund & Beckwith, 1999b). However, it can be replaced by other thioredoxins or glutaredoxins. In *B. subtilis*, no backup system is known and TrxA is indispensable for growth under the conditions tested. To determine whether *B. subtilis* cells respond to TrxA depletion at the level of protein synthesis or not, we analyzed the proteome of IΔ*trxA* after depletion of TrxA. Therefore, protein extracts of IΔ*trxA* cells 45, 90, 135 and 170 minutes after exchange of the medium were separated by means of 2D gel electrophoresis. The change in protein expression pattern was visualized by dual-
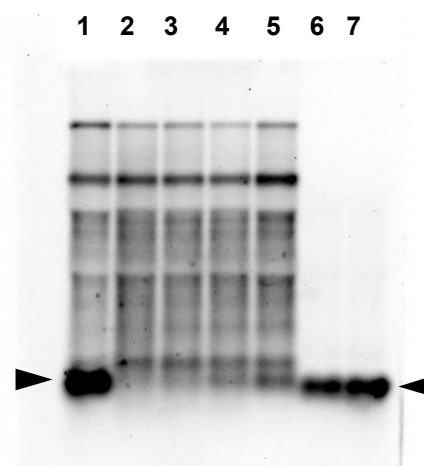

Fig. 2. Northern analysis of IΔ*trxA* in synthetic medium before and after depletion of IPTG. RNA was prepared from cells growing with 50 µM IPTG (*lane 1*) or without IPTG (*lane 2-5*). Lanes 2-5 represent RNA preparation from cells harvested 45, 75, 135 and 195 min after transferring the cells to medium without IPTG. For comparison, in lane 6 and 7, RNA of exponentially growing *B. subtilis* wild-type cells were probed. Electrophoretic separation of the RNA (5 µg per lane) was carried out using 1.2% gels. *trxA* transcript is indicated by *arrows*.
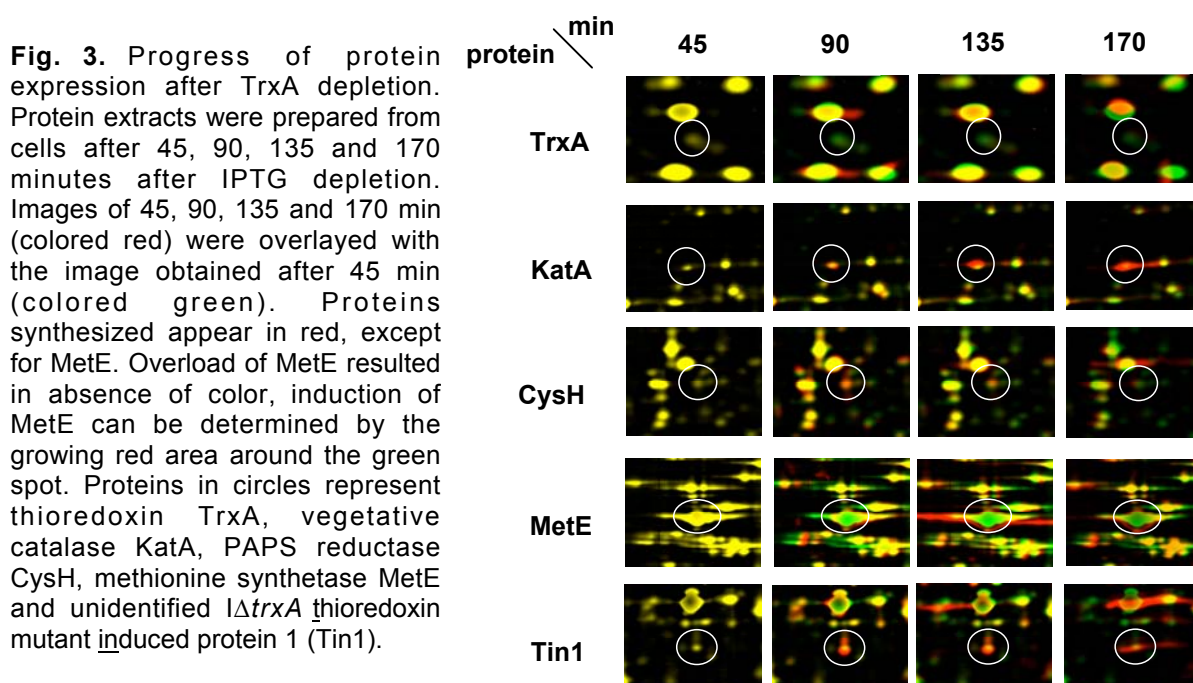
channel imaging. Therefore, images generated by protein pattern of the three latter time points were overlaid with the image obtained from the first sample (Fig. 3). TrxA is only present in cells that were harvested at 45 minutes after transferring the cells to IPTG-free medium indicating a downregulation by IPTG depletion. Several proteins were found in larger amounts after 90, 135 or 170 minutes. Within this group, catalase KatA, methionine synthase MetE, and PAPS reductase CysH were identified. Whereas induction of KatA indicates oxidative stress in the absence of the antioxidant TrxA, it is interesting to note that the unspecific DNA-protecting protein MrgA was not observed at significant

elevated expression levels. Interestingly, Auger et al. (2002) reported increased transcription of *katA* but not of *mrgA* when *B. subtilis* wild-type cells were grown in the presence of methionine. These authors suggested that providing methionine as the sole sulfur source represented sulfur-limitation.

The upregulation of two proteins belonging to the sulfate-assimilation pathway in *B. subtilis* might indicate an increased demand of sulfur or a sulfur limitation in the absence of TrxA. In *E. coli*, thioredoxin is responsible for the re-reduction of PAPS reductase which undergoes a thiol-disulfide exchange reaction thereby reducing sulfate to sulfite (Holmgren, 1989). This could also be the case for *B. subtilis* thioredoxin. Since we used a synthetic medium containing $MgSO_4$, $FeSO_4$ and $MnSO_4$, the sole sulfur source for *B. subtilis* cells was sulfate. In *B. subtilis*, inorganic sulfate is suggested to be reduced to sulfide by a sequence of enzymic steps including reduction of PAPS to sulfite carried out by PAPS reductase CysH (Mansilla & de Mendoza, 1997; Mansilla et al., 2000). If *B. subtilis* TrxA is involved in reactivation of PAPS reductase and *B. subtilis* contains no backup systems for re-reducing PAPS reductase, the cells would experience sulfur limitation under the tested conditions.

Altogether, the data obtained indicated limitation of sulfur when IΔ*trxA* cells are grown in sulfate-containing synthetic

Fig. 3. Progress of protein expression after TrxA depletion. Protein extracts were prepared from cells after 45, 90, 135 and 170 minutes after IPTG depletion. Images of 45, 90, 135 and 170 min (colored red) were overlayed with the image obtained after 45 min (colored green). Proteins synthesized appear in red, except for MetE. Overload of MetE resulted in absence of color, induction of MetE can be determined by the growing red area around the green spot. Proteins in circles represent thioredoxin TrxA, vegetative catalase KatA, PAPS reductase CysH, methionine synthetase MetE and unidentified IΔ*trxA* thioredoxin mutant induced protein 1 (Tin1).

medium and when the cells are depleted of TrxA protein.

Analysis of sulfur sources. To address the question whether thioredoxin of *B. subtilis* is indeed essentially involved in sulfate assimilation, growth experiments with alternative sulfur sources were carried out. *B. subtilis* wild-type cells were tested for their ability to grow on various sulfur sources in the synthetic medium we used. Methionine and thiosulfate (final concentration 1.0 mM) were found to represent sulfur sources that, when replacing sulfate, led to comparable growth rates to the growth behavior with sulfate. When the medium was exchanged against medium without any sulfur source, growth of *B. subtilis* wild-type cells ceased, indicating that no sulfur-containing contaminants were present in the medium or in supplements (data not shown).

Cells of *B. subtilis* I∆*trxA* were grown in MgSO₄-containing medium with IPTG to the late exponential phase. To replace the sulfur source, cells were centrifuged and resuspended in synthetic medium containing Na-thiolsulfate or methionine and cell growth was monitored (Fig. 4A, B). For supplementation with both methionine and thiosulfate as the sole sulfur source, no impairment with or without IPTG was observed. The obtained results indicate that in contrast to growth with sulfate TrxA appears to be dispensable for growth with
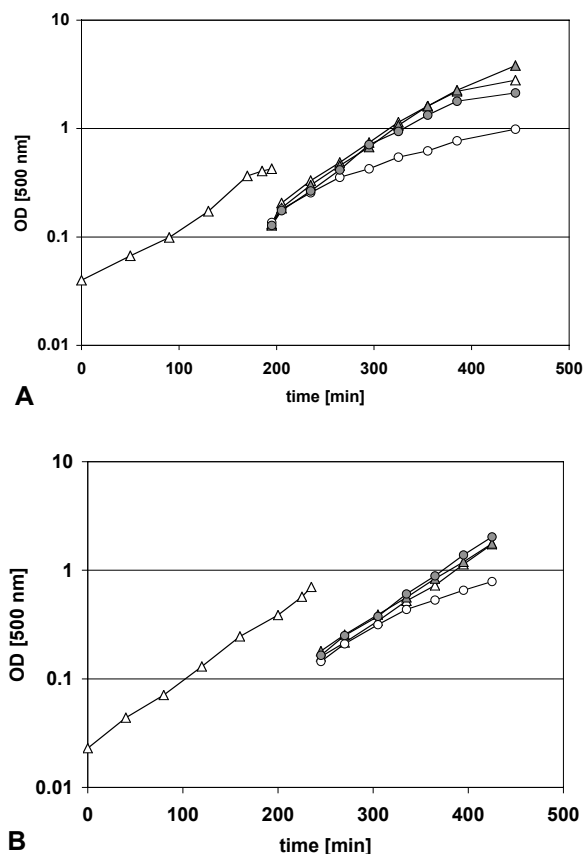

Fig. 4. Growth monitoring of *B. subtilis* I∆*trxA* with methionine (**A**) or thiosulfate (**B**) as sulfur sources. Cells were grown exponentially in the presence of sulfate and IPTG (*open triangles*). Medium was replaced by synthetic medium containing sulfate and IPTG (*open triangles*) or sulfate without addition of IPTG (*open circles*), methionine and IPTG (**A:** *gray triangles*) or methionine without IPTG (**A:** *gray circles*), or thiosulfate with IPTG (**B:** *gray triangles*) or thiosulfate without IPTG (**B:** *gray circles*).

methionine or thiolsulfate. Furthermore, it can be suggested temptatively, that *B. subtilis* thioredoxin is only essential for sulfate assimilation but not in relation to other functions under conditions of exponential growth. However, preliminary data indicated that I∆*trxA* is transiently impaired in exponential growth when sulfite is added instead of sulfate. Because TrxA

may re-reduce PAPS reductase CysH in *B. subtilis*, sulfite should represent a sulfur source that can be assimilated also in the absence of TrxA.

The protein expression pattern in IΔtrxA after 170 minutes of growth with thiosulfate were compared to protein expression pattern obtained by growth with sulfate when cells were depleted of TrxA (Fig 5). In the presence of sulfate, KatA, MetE, CysH and unidentified proteins (Tin) were detected on significantly higher expression levels than in cells that were grown in the presence of thiosulfate. This result underlines the previous suggestion that TrxA is essentially involved in assimilation

of sulfate but dispensable for growth with other sulfur sources incorporated by alternative pathways. Interestingly, in these experiments also increased levels of cysteine synthetase CysK, a protein involved in cysteine biosynthesis from sulfide and o-acetylserine and peroxide specific AhpF and AhpC were detected to be at higher expression level. These proteins were not detected in previous attempt indicating some variation in protein synthesis.

Conclusion

The thiol-disulfide oxidoreductase thioredoxin is an essential protein in

Fig. 5. Protein amounts of IΔ*trxA* after 170 minutes of growth without IPTG in the presence of sulfate (colored orange) and in the presence of thiosulfate (colored blue) as sole sulfur sources. Proteins indicated are present in higher amounts when cells were grown on sulfate.
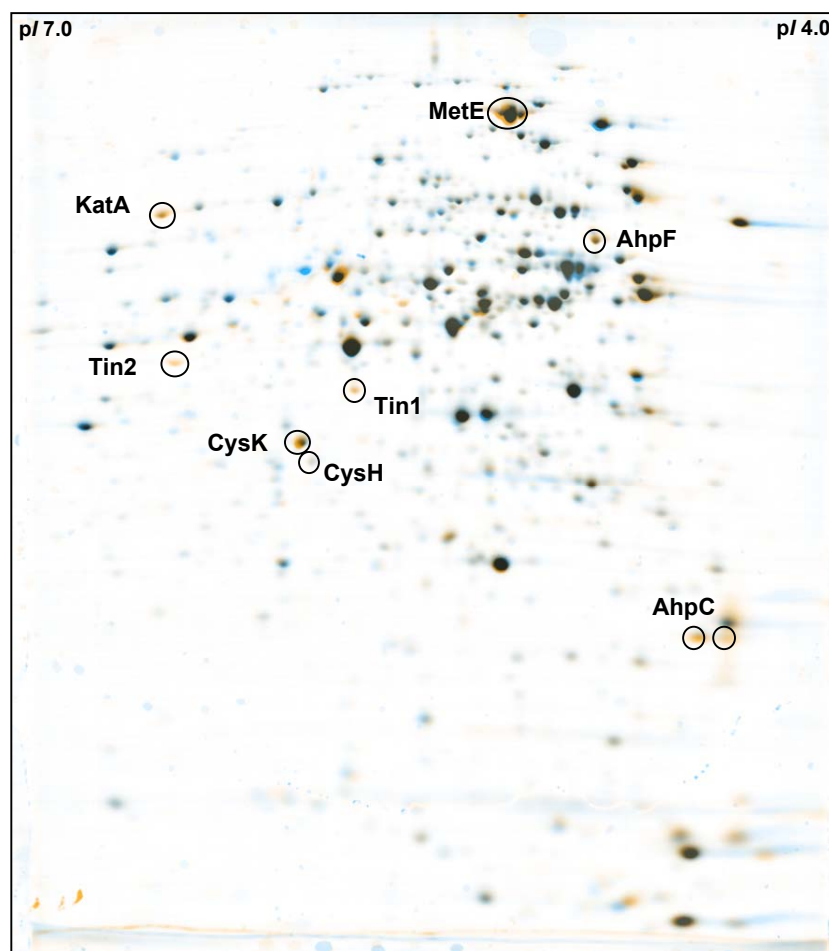
B. subtilis (Scharf et al., 1998). Analysis of the conditional mutant I∆*trxA* by means of growth monitoring and 2D gel electrophoresis indicated a sulfur limitation when cells were depleted of TrxA. This suggests that thioredoxin is indispensable for sulfate assimilation in *B. subtilis*. In *E. coli*, thioredoxin is known to be involved in re-reduction of PAPS reductase as part of the sulfate assimilation pathway (Holmgren, 1989). When sulfate was replaced by methionine or thiosulfate in this work, the impairment of growth observed for I∆*trxA* with sulfate as the sole sulfur source was abolished underlining our hypothesis. Furthermore, I∆*trxA* cells grown in the presence of thiosulfate did not respond by induction of catalase or proteins involved in sulfur-assimilation at the level of protein synthesis. However, we cannot exclude protective or antioxidant functions of thiosulfate or methionine leading to the observed phenotypical characteristics. The next steps in functional analysis of *B. subtilis* thioredoxin should include construction of a null mutant in *trxA* whereby sulfate has to be replaced by thiosulfate or methionine as the sole sulfur source.

General Discussion

In all four parts of the present work, aspects of the response of *B. subtilis* cells to increased levels of reactive oxygen species (ROS) or to decreased levels of antioxidants were analyzed. While in the first part the change of gene expression pattern in response to superoxide (O_2^-) and hydrogen peroxide (H_2O_2) was investigated, the experiments in the second and third part focused on the role of the general stress response in resistance to oxidative stress. In the fourth part, the physiological function of the thiol-disulfide oxidoreductase TrxA of *B. subtilis* was investigated.

In addition to the identification of major responsive elements activated after O_2^- and H_2O_2 stress that was demonstrated in the first part, also a slight induction of σ^B-dependent genes was observed. From the results obtained, we concluded that in contrast to the induction of the PerR regulon the weak induction of the σ^B-regulon is of minor physiological importance when additional limitations or stress stimuli are absent.

In the second part, the level of protein carbonyl formation after peroxide stress was analyzed especially with respect to a putative involvement of the general stress response. We inferred that the observed protection against protein carbonylation under conditions of glucose starvation might be a consequence of the action of coinduced peroxide specific proteins but is not an effect of the general stress response. The demonstration that *B. subtilis* cells were protected against protein carbonylation when expression of σ^B was activated using an artificial induction system, led us to the conclusion that the general stress response might indeed have impact to protect cells from protein damage after oxidative stress, but under altered conditions.

In the third part, we demonstrated that cross-protection by σ^B is not limited to glucose starved cells, but is also valid for cells pretreated by transient heat shock when moderate H_2O_2 concentrations were used. By a screening of strains with mutations in σ^B-dependent genes, a strain carrying a mutation in the *dps* gene was identified to resemble the phenotype of a *sigB* mutant strain after peroxide treatment. The results obtained by these analyses led us to the assumption that the general stress response provides primarily DNA protection under the tested conditions.

The fourth part focused on the investigation of the thiol-disulfide oxidoreductase thioredoxin. From the obtained results of growth monitoring and 2D gel electrophoresis we suggested that TrxA is essentially involed in re-reduction of PAPS reductase CysH. In addition to initiation of transcription by RNA polymerase containing the vegetative sigma

factor σ^A, the gene encoding TrxA is also subject to σ^B-dependent transcription (Scharf et al., 1998).

Altogether, the data presented in this work underlay the assumption that σ^B is involved in the protection against increased levels of ROS under conditions of additional limitations or stresses. It may be suggested that the general stress response and the peroxide-specific stress response of *B. subtilis* are carefully integrated into the regulatory network as both provide antioxidant functions. Interestingly, a similar overlap is observed for the response network of *E. coli*, indicating a major physiological importance for multiple antioxidant modules in aerobically growing organisms (Storz & Zheng, 2000).

Specific stress response in *E. coli* and *B. subtilis*

In *E. coli*, the adaptive response to ROS is under the control of the OxyR and SoxRS regulatory proteins. After treatment with H_2O_2, the LysR-type regulatory protein OxyR is activated by direct oxidation triggering an intramolecular thiol-disulfide switch of cysteine residues (Zheng et al., 1998). In its oxidized form, OxyR binds four adjacent major groove regions of target DNA and activates transcription by direct contact with RNA polymerase. The OxyR regulon includes many genes encoding proteins with clear antioxidant roles, e.g. catalase KatG, alkyl hydroperoxide reductase AhpFC, nonspecific DNA binding protein Dps, glutathione reductase GorA etc. (Zheng et al., 2001). In addition, the transcription of important regulators, i.e. the *fur* gene encoding the ferric uptake repressor Fur and the small RNA *oxyS*, is controlled by OxyR (Altuvia et al., 1997; Zheng et al., 1999). In response to O_2^- challenge, the O_2^- specific SoxRS regulon is induced by a two-stage activation process of the SoxRS protein complex (Gaudu et al., 1997; Hidalgo et al., 1997). First, an oxidation of the $[2Fe-2S]^+$ cluster to $[2Fe-2S]^{+2}$ activates the SoxR regulatory protein. SoxR in its active form enhances transcription of *soxS* mRNA encoding SoxS regulatory protein that in turn activates expression of the whole regulon. Within the SoxRS regulon proteins with understood roles in the defense against O_2^- generating agents have been identified, e.g. superoxide dismutase SodA and glucose-6-phosphate dehydrogenase encoded by the *zwf* gene (Pomposiello et al., 2001). Furthermore, expression of O_2^--resistant isozymes of fumarase and aconitase have been observed to be at elevated levels (Liochev & Fridovich, 1992; Gruer & Guest, 1994). The *fur* gene was also found at higher expression levels in a SoxRS-dependent manner in response to O_2^- stress (Zheng et al., 1999). Besides the SoxRS regulon, the OxyR regulon was as well induced after treatment with O_2^- generating agents (Pomposiello et al., 2001).

A comparison between the changes of gene expression pattern of *B. subtilis* and *E. coli* cells in response to ROS treatment revealed striking similarities with respect to the peroxide specific stress response but not to the superoxide specific stress response. Despite the fact that PerR of *B. subtilis* and OxyR of *E. coli* show no homologies to each other and differ in the mode of operation (Mongkolsuk & Helmann, 2002), i.e. transcriptional repression *versus* transcriptional activation, the set of proteins included in both regulons is very similar. Moreover, the regulons are induced in response to H_2O_2 treatment and after addition of O_2^- generating agents.

Besides genes encoding products with antioxidant roles that are included in both regulons, e.g. catalases, alkyl hydroperoxide reductases and non-specific DNA binding proteins, in *E. coli* as well as in *B. subtilis* the *fur* gene encoding ferric uptake repressor Fur is observed to be regulated by the respective peroxide specific regulator. The fact that in both organisms the *fur* gene is under control of oxidative stress specific regulators indicates a crucial link between the levels of intracellular iron and the occurrence of ROS in aerobic bacteria. Whether the derepression of the Fur regulon observed in *B. subtilis* is of physiological significance or if the synthesis of Fur indicates the need for iron-sequestration as it was suggested for Fur of *E. coli* is an important question that has to be addressed in future studies.

No O_2^- specific response was identified in *B. subtilis* cells, in contrast to the situation in *E. coli*. Moreover, no induction of the putative superoxide dismutase SodA was observed in response to O_2^- stress under the conditions tested. The separate regulation of antioxidant activities in *E. coli* in response to O_2^- and H_2O_2 is suggested to be very efficient. For *B. subtilis*, we do not know, to which extend supplements of the medium used might had an influence on O_2^- dismutation since it was reported that Mn(II) can dismutate O_2^- with a Sod mimicking activity (Inaoka et al., 1999). Thus, the presented results can only represent a first step for a more detailed investigation of the O_2^- stress response of *B. subtilis* cells.

General stress response in *E. coli* and *B. subtilis*

As already shown in earlier studies, the peroxide specific stress response and the general stress response show a functional overlap for resistance to ROS in *B. subtilis* (Antelmann et al., 1996; Engelmann & Hecker, 1996). Pretreatment of *B. subtilis* cells with low concentrations of H_2O_2, by transient heat stress or under conditions of glucose starvation confers increased resistance to the exogenous addition of H_2O_2. The increased resistance

after peroxide pretreatment is mediated by the peroxide specific stress response while the latter two are dependent on the activation of the general stress response. Interestingly, resistance after transient heat stress was shown to be limited to moderate concentrations of H_2O_2 while glucose limitation was reported to confer resistance to otherwise lethal concentrations of H_2O_2 (Engelmann & Hecker, 1996).

A comparison of the PerR controlled peroxide specific regulon and the σ^B-dependent general stress regulon reveals homologies between several gene products. Both regulons contain at least one gene encoding a functional catalase. The gene for the vegetative catalase KatA is induced by derepression of the PerR regulon and was observed to show one of the highest levels of induction on mRNA level. Within the σ^B-regulon, two genes encoding catalase enzymes were identified (Engelmann et al., 1995; Petersohn et al., 1999a). While the gene encoding KatE is dependent on σ^B, the gene *katF* is subject to double control by σ^B and the sporulation forespore-specific sigma factor σ^F. In addition, both regulons control the expression of a non-specific DNA binding protein *dps* encoding a member of the Dps/PexB-protein family (Chen & Helmann, 1995; Antelmann et al., 1997a). Similar to the *katA* gene, the PerR regulated gene *mrgA* displayed one of the highest transcriptional induction levels after oxidative stress. The gene encoding Dps is preceded by promoters for σ^A- and σ^B-dependent initiation of transcription. Induction of *dps* is predominantly ascribed to the σ^B-dependent promoter (Antelmann et al., 1997a). The PerR regulon as well as the σ^B-regulon include gene products that provide resistance to organic hydroperoxides. The AhpC subunit of the alkyl hydroperoxide reductase AhpCF reduces organic peroxides to the corresponding alcohols (Antelmann et al., 1996; Bsat et al., 1996). AhpC is re-reduced by the NADPH-dependent reductase AhpF. The genes encoding AhpC and AhpF are organized in a single operon controlled by PerR (Bsat et al., 1998). Recently, a new type of organic hydroperoxide resistance protein was identified in *Xanthomonas campestris* (Mongkolsuk et al., 1998). In the following, we recognized that the OsmC-homologue *ykzA* of *B. subtilis*, which was shown to be regulated by σ^B, is involved in resistance to organic hydroperoxides (Völker et al., 1998; Fuangthong et al., 2001). Altogether, numerous genes encoding proteins with putative antioxidant function are included in the general stress regulon and are induced by σ^B. This suggests that the general stress response provides diverse antioxidant strategies, e.g. detoxification of inorganic and organic peroxides and non-specific DNA protection. However, specific targets of oxidative damage were not identified in *sigB* mutant cells up till now.

Similar to the situation observed in *B. subtilis*, *E. coli* cells display an overlap between

the OxyR-dependent stress response and the stationary phase regulon controlled by stationary phase sigma factor σ^S (Ivanova et al., 1997). While in *B. subtilis* different sets of proteins are induced by PerR inactivation and σ^B activation, in *E. coli*, genes encoding proteins with antioxidant functions, e.g. *katG*, *dps* and *gorA* are under control of OxyR and RpoS (Storz & Zheng, 2000). In addition, σ^S regulates the expression of several antioxidant genes, e.g. *katE* and *xthA* encoding catalase HPII and exonuclease III respectively (Eisenstark et al., 1996). Recently, the σ^S-regulated periplasmic CuZn superoxide dismutase was identified to be involved in H_2O_2 resistance (Gort et al., 1999). Interestingly, it was demonstrated for *E. coli* that the gene encoding OxyR is under control of σ^S (Michan et al., 1999). In the contrary, the *oxyS* RNA, which is under positive control of OxyR, acts to repress translation of *rpoS* mRNA (Zhang et al., 1998). The similarity between the functional organization of antioxidant regulation in *B. subtilis* and *E. coli* indicates the importance of antioxidant activities in aerobic organisms under various environmental conditions. Since it was suggested that the general stress response confers the cells resistance to future stress, the results of our group obtained up till now led to the suggestion that *B. subtilis* cells likewise to *E. coli* might survive extended periods of stress and starvation in their natural environments in form of vegetative cells. The equipment with several antioxidant activities organized in different response modules supports the idea that oxidative stress is one of the major environmental threats in aerobically living bacteria that fluctuate between growing and non-growing states.

The role of Dps in *E. coli* and *B. subtilis*

In this work, we presented i) that the σ^B-dependent induction of the general stress response did not suffice to prevent oxidative modifications of proteins and ii) that a mutant strain of the *dps* gene encoding the non-specific DNA binding protein Dps resembles phenotypical characteristics of the *sigB* mutant strain after oxidative stress. Together these results indicate that the general stress response confers non-specific protection of DNA after H_2O_2 challenge in *B. subtilis* cells. Other mechanisms, e.g. detoxification of H_2O_2 or degradation of oxidatively damaged proteins, both possibly visible by means of minimized levels of protein carbonyls, appear to be of minor physiological importance under the tested conditions. Our observation support earlier findings concerning the involvement of Dps but not catalase KatE in oxidative stress resistance in glucose starved cells (Engelmann & Hecker, 1996; Antelmann et al., 1997a).

In *E. coli*, Dps is assumed to protect DNA by biocrystallization, i.e. Dps and DNA

interact and form extremely stable crystals (Wolf et al., 1999). Transcription of the *dps* gene is induced in response to oxidative stress and in the stationary phase. Martinez and Kolter (1997) demonstrated that the *E. coli* Dps protein protects DNA by direct and unspecific binding rather than activating a repair mechanism. A similar mode of operation might be true for *B. subtilis* Dps. However, since the homology between Dps of *E. coli* and Dps of *B. subtilis* is only weak, the function in *B. subtilis* still has to be confirmed. Recently, it was reported that the crystal structure of Dps from *Bacillus brevis* reveals the structural basis for the proposed ferroxidase activity of the protein. Thus, the enzymic detoxification of metal ions, which may cause severe damage in the vicinity of DNA after oxidative stress, was shown to be another highly important aspect of Dps (Ren et al., 2003).

In this work, an attempt was done to prove the putative damage of DNA in *sigB* mutant cells in comparison to wild-type cells by means of terminal deoxyribonucleotide transferase (TdT)-mediated dUTP nick end labeling (TUNEL) but it failed to reveal increased levels of damaged DNA (data not presented), most probably because of methodical problems. The TUNEL assay is primarily designed to analyze apoptosis induced fragmentation of eukaryotic DNA, i.e. increase of double strand brakes, and the enzyme used might have failed to label DNA termini of single strand brakes. In *E. coli* and the archaebacterial species *Haloferax volcanii*, the assay was carried out with success but only after exposing the cells to extremely high conditions of H_2O_2 (Rohwer & Azam, 2000). To clarify if DNA of *sigB* mutant cells is indeed subject to increased damage, we suggest the analysis of the spontaneous mutation frequency in wild-type, $\Delta sigB$ and Δdps *B. subtilis* cells under conditions of prolonged glucose starvation and transient heat stress in combination with exposure to peroxide. Since oxidative stress leads to mutagenesis, DNA-protection deficient strains might accumulate mutations earlier than wild-type cells. In addition, experiments that elucidate the role of iron ions after oxidative stress concerning DNA damage and the involvement of the general stress response herein are of future interest.

The general stress response of σ^B and its function in aerobic life

The σ^B group of sigma factors is restricted to a specific subgroup of Gram-positive bacteria including *Staphylococcus* and *Listeria* species and is related to sigma factors of *Mycobacterium tuberculosis* and *Streptomyces* and *Synechocystis* species (Mittenhuber, 2002). Besides stress response related functions reported for the σ^B group, the σ^B-related sigma factors of *M. tuberculosum*, *Synechocystis* sp. PCC6803 and *Streptomyces coelicolor* are involved in providing stress resistance (DeMaio et al., 1996; Huckauf et al.,

2000; Kormanec et al., 2000). From the finding that i) σ^B of *B. subtilis* is essentially involved in stationary phase as well as heat shock mediated resistance to peroxides and ii) σ^B related proteins are absent in strictly anaerobic Gram-positive *Clostridia* species, it was assumed that the presence of ROS might have even supported the evolution of the general stress response (Hecker & Völker, 2001). Recently, it was demonstrated that σ^B of *Listeria monocytogenes* also provides resistance to oxidative stress (Ferreira et al., 2001). In *M. tuberculosum*, the gene encoding the σ^B-related sigma factor is under control of SigH. Since a *sigH* mutant is more sensitive to various oxidative stresses, it is tempting to speculate that the σ^B-related sigma factor could be involved in direct regulation of genes to provide oxidative stress resistance. Whether the adaptive response concept suggested for the general stress response of *B. subtilis* is conserved in *Bacillus*, *Staphylococcus* and *Listeria* species and a very similar concept is distributed over a large group of aerobically Gram-positive bacteria is a question that will be answered in the near future. Due to the powerful tools of genome sequencing and transcriptome analysis, data about various Gram-positive bacteria will become available that have to be analyzed by informatic approaches. Analysis of these data will also contribute to the progress of elucidating the role of numerous general stress proteins to which no function could be assigned up till now.

Conclusion

By the combination of gene expression pattern analysis with the determination of oxidative damage of proteins and stress protection experiments, the presented work includes a broad investigation of *B. subtilis* specific and general strategies in response to oxidative stress. It provides a global view of two essential modules of the regulatory response network in *B. subtilis* that confer resistance to oxidative stress. For the complete understanding of the integration of the σ^B- and PerR-dependent stress responses into the regulatory network further analysis would be of interest. The question has to be addressed under which natural conditions *B. subtilis* cells have to face oxidative stress by an exogenous or endogenous increase of ROS.

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List of Publications

Mostertz, J. & Hecker, M. (2003). Patterns of protein carbonylation following oxidative stress in wild-type and *sigB Bacillus subtilis* cells. *MGG* **269**, 640-648.

Gerth, U. , Kirstein, J., Mostertz, J., Waldminghaus, T., Miethke, M., Kock, H., & Hecker, M. (2004). Fine-tuning in regulation of Clp protein content in *Bacillus subtilis*. *J Bact* **186**, 179-191.

Mostertz, J., Scharf, C., Hecker, M. & Homuth, G. (2004). Transcriptome and proteome analysis of *Bacillus subtilis* gene expression in response to superoxide and peroxide stress. *Microbiol* **150**, 497-512.

Eidesstattliche Erklärung

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Ernst-Moritz-Arndt-Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich, dass ich diese Arbeit selbständig verfasst und keine anderen als die darin angegebenen Hilfsmittel benutzt habe.

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