

## Diamide Triggers Mainly S Thiolations in the Cytoplasmic Proteomes of *Bacillus subtilis* and *Staphylococcus aureus*<sup>∇†</sup>

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**Glutathione constitutes a key player in the thiol redox buffer in many organisms. However, the gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* lack this low-molecular-weight thiol. Recently, we identified S-cysteinylated proteins in *B. subtilis* after treatment of cells with the disulfide-generating electrophile diamide. S cysteinylated proteins are thought to protect protein thiols against irreversible oxidation to sulfenic and sulfonic acids. Here we show that S thiolation occurs also in *S. aureus* proteins after exposure to diamide. We further analyzed the formation of inter- and intramolecular disulfide bonds in cytoplasmic proteins using diagonal nonreducing/reducing sodium dodecyl sulfate gel electrophoresis. However, only a few proteins were identified that form inter- or intramolecular disulfide bonds under control and diamide stress conditions in *B. subtilis* and *S. aureus*. Depletion of the cysteine pool was concomitantly measured in *B. subtilis* using a metabolomics approach. Thus, the majority of reversible thiol modifications that were previously detected by two-dimensional gel fluorescence-based thiol modification assay are most likely based on S thiolations. Finally, we found that a glutathione-producing *B. subtilis* strain which expresses the *Listeria monocytogenes gshF* gene did not show enhanced oxidative stress resistance compared to the wild type.**

Cysteine thiols in proteins fulfill an important and diverse set of cellular functions. In particular, they participate in enzymatic catalysis; in metal coordination, such as in the generation of Fe-S-clusters; and in determining the spatial structure of proteins via disulfide bond formation (3, 22, 23, 38). Cysteines are strong nucleophiles amenable to posttranslational modifications by reactive oxygen species (ROS) and reactive nitrogen species, leading to disulfides; to sulfenic, sulfenic, or sulfonic acids; mixed disulfides with low-molecular-weight (LMW) thiols (S thiolations); and S nitrosylations (7, 16, 17, 27).

The redox status of the cytoplasm is under physiological conditions in a reduced state. Thus, most cysteines are present as free thiols (6). Because aerobic organisms have to cope with oxidative stress caused by ROS, such as superoxide anions, hydrogen peroxide, or hydroxyl radicals, they need to employ effective mechanisms that maintain the reduced state. In gram-negative bacteria, the thiol-disulfide balance is accomplished by the glutathione (GSH) system, a thiol-based redox buffer. The GSH system consists of glutaredoxin (Grx), GSH ( $\gamma$ -glutamylcysteinyl glycine), GSH reductase, and GSH peroxidase

(34). Reduction of disulfides occurs via sequential electron transfer from glutaredoxin and reduced GSH; oxidized GSH (GSSG) is reduced by the NADPH-dependent GSH reductase. GSH peroxidase enables the direct detoxification of ROS by GSH oxidation.

However, many gram-positive bacteria lack genes for GSH biosynthesis. Actinomycetes instead use a thiol redox buffer based on mycothiol (50). *Bacillus subtilis*, *Staphylococcus aureus*, and other gram-positive bacteria rely on different thiol redox buffers based on cysteine, the novel 398-Da bacillithiol (BSH), or coenzyme A (CoA) (15, 52). To maintain the reduced state of the cytoplasm, most bacteria use enzymatic systems for disulfide bond reduction such as the thioredoxin (Trx) system, which is highly conserved in gram-negative bacteria (3, 10). The Trx system consists of thioredoxin (TrxA) and the NADPH-dependent thioredoxin reductase (TrxB).

Any imbalance in the cellular redox state caused by ROS elicits expression of a repertoire of different proteins, commonly under the control of a redox-sensing regulator: for example, OxyR in *Escherichia coli* and PerR, OhrR, SarZ, and Spx in *B. subtilis* and *S. aureus*, respectively (11, 12, 41, 55, 58, 64–66). The subsequently induced proteins detoxify ROS and restore and protect the normal physiological redox state in the cell.

Besides ROS and reactive nitrogen species, so-called “reactive electrophilic species” (RES) affect the thiol redox balance. RES include different chemical compounds such as aldehydes, quinones, and the azo compound diamide (2, 43, 45, 46, 53, 66). Quinones and aldehydes have electron-deficient centers that result in thiol(-S) alkylation of cysteine. Exposure of cells to diamide induces the oxidative as well as the electrophile

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stress response in *B. subtilis* (43, 45, 53). The toxicity of diamide is based on disulfide bond formation (40), which was recently visualized in *B. subtilis* and *S. aureus* by the fluorescence alkylation of oxidized thiols (FALKO) assay (32, 64). It was thought that the formation of nonnative inter- and intramolecular disulfide bonds results in damage of proteins.

However, more recent findings demonstrate that diamide stress leads also to S thiolations: formation of disulfide bonds between proteins and LMW thiols (8, 13, 33). S thiolations prevent protein thiols from irreversible oxidation to sulfinic and sulfonic acids, but also affect enzyme activity (35, 47) and signal transduction (39, 42). In *B. subtilis*, we have identified a few cytoplasmic proteins that are S cysteinylated (33). In addition, the organic peroxide sensor OhrR was inactivated by an S bacillithiolation in *B. subtilis* (42).

Cysteine, BSH, and CoA are also dominant LMW thiols in *S. aureus* (52). In this study, we have investigated in more detail the extents of S thiolations and inter- and intramolecular disulfide bond formation of *B. subtilis* and *S. aureus* in response to disulfide stress. The results showed that exposure to diamide leads to S thiolations in *S. aureus*. Using a nonreducing/reducing sodium dodecyl sulfate (SDS) diagonal electrophoresis approach, proteins with intermolecular disulfide bonds could be distinguished from proteins with intramolecular disulfide bonds (57). The results support that the majority of reversible thiol oxidations are based on S thiolations rather than disulfide bonds between proteins. Depletion of the free cysteine pool in *B. subtilis* after exposure to diamide supports this finding. To assess if GSH may have a bearing on the thiol redox buffer of *B. subtilis*, the *gshF* gene of *Listeria monocytogenes* (*gshF<sub>LM</sub>*) was expressed in *B. subtilis*, enabling GSH biosynthesis (29). Although GSH production does not enhance the resistance to oxidative stress in *B. subtilis*, it participates in the formation of S thiolations.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The bacterial strains used were *B. subtilis* 168 (*trpC2*) (1) and a *B. subtilis* strain expressing *gshF<sub>LM</sub>* (*trpC2 amyE::lacI gshF Spec<sup>r</sup>*). All *B. subtilis* strains were cultivated in a synthetic medium (60) at 37°C under vigorous agitation. *S. aureus* UAMS-1 (28) was cultivated in a synthetic medium described earlier (26). However, MOPS (morpholinepropanesulfonic acid) and glycine were omitted and all amino acids were at 1 mM. If needed, cysteine was substituted by 1 mM Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>. Spectinomycin was used for *B. subtilis gshF<sub>LM</sub>* at 100 µg/ml. GSH production in the *B. subtilis gshF<sub>LM</sub>* strain was induced with 40 µM IPTG (isopropyl-β-D-thiogalactopyranoside) at an optical density at 500 nm (OD<sub>500</sub>) of 0.2. All strains were grown to the exponential growth phase (OD<sub>500</sub> of 0.5) and exposed to 1 mM (*B. subtilis*) or 2 mM (*S. aureus*) diamide. The *B. subtilis gshF<sub>LM</sub>* strain was exposed to 50 µM Paraquat and 100 µM H<sub>2</sub>O<sub>2</sub>. Controls were taken at an OD<sub>500</sub> of 0.5.

**Growth curves, survival tests, and DCW.** Growth curves were performed by measuring OD<sub>500</sub> at different time points. For survival tests, samples from bacterial cultivations were diluted and 100 µl was plated onto LB agar plates. The plates were incubated over night at 37°C, colonies were counted, and subsequently the weighted arithmetic mean was determined. For calculations of the dry cell weight (DCW), 20 ml of *B. subtilis* cells was collected by centrifugation at different time points as replicates. At these time points, the OD<sub>500</sub> was measured with different dilutions of the culture. The cells were washed twice with distilled water and transferred to a constant-pretreated glass vial. The glass vial was dried in an oven (60°C) to constant weight. The OD<sub>500</sub> was plotted against the net weight of the glass vials. The analysis of the regression resulted in the following relationship: DCW (mg/ml culture) = 0.2107 × OD<sub>500</sub> + 0.0043, with R<sup>2</sup> = 0.995.

**Construction of the *B. subtilis gshF<sub>LM</sub>* strain.** A 2.43-kb fragment of *Listeria monocytogenes* EGD-e DNA, containing the *gshF* gene (lmo2770) including its

putative ribosomal binding site, was amplified by PCR employing a forward SphI primer, 5'-TATAGCATGCTTATTAAACCCCTGAGGTG-3', and a reverse SphI primer, 5'-ATATGCATGCAAATGGTGAAATTGGATTG-3'. The underlined letters show the SphI restriction site. The purified fragment digested with SphI was sequenced to verify identity to the deposited sequence and cloned into SphI sites of the integration vector pDR79 (*amyE::PspA-C-*spc**; a gift from Sigal Ben-Yehuda, The Hebrew University, Jerusalem, Israel). The resulting construct, containing a proper *PspA-C-gshF* fusion, was designated pDR79-*gshF*. A linearized pDR79-*gshF* DNA was transformed into competent *B. subtilis* 168 cells and integrated into the *amyE* gene. Transformants were selected for spectinomycin resistance (100 µg/ml) and designated the *B. subtilis gshF<sub>LM</sub>* strain, which contained a disrupted *amyE* gene with an intact GSH synthase gene fused to the IPTG-inducible *PspA-C* promoter.

**HPLC analysis of LMW thiols from *B. subtilis*.** Cells were cultured as described above. Two milliliters of culture was centrifuged for 1 min and washed with 50 mM Tris-HCl (pH 8.0). The pellet was resuspended in 50 µl extraction buffer (50% [vol/vol] acetonitrile [ACN] in 20 mM Tris-HCl, pH 8.0) containing 2 mM monobromobimane (mBB; Molecular Probes). As an internal standard, 1 µM penicillamine was added to each sample. The suspension was incubated for 15 min at 60°C in the dark. Control samples were incubated for 10 min with 5 mM *N*-ethylmaleimide (NEM; Sigma-Aldrich) under the same conditions before the addition of mBB (2 mM). The cellular debris was removed by centrifugation, and the reaction was stopped by addition of 5 mM aqueous methane sulfonic acid. Thiol standards were prepared as described previously (21).

A derivatized sample was separated by high-performance liquid chromatography (HPLC) (51), and peak recognition was performed with a fluorescence detector (Shimadzu RF-10AXL). Briefly, the CoA method was used, which constituted a tetrabutylammonium phosphate (TBAP; Sigma-Aldrich) ion-pairing protocol designed for the separation of CoA-bimane derivatives (51). A C<sub>8</sub> RP column (AccQ-Tag, 3.0 µm, 150 mm; Waters) at a flow rate of 0.8 ml min<sup>-1</sup> was used in a column oven at 25°C. The chromatographic protocol employed the following solvents and gradients: solvent A, 10% (vol/vol) methanol, 0.25% (vol/vol) acetic acid, and 10 mM TBAP, pH 3.4; solvent B, 90% (vol/vol) methanol, 0.25% (vol/vol) acetic acid, and 10 mM TBAP. At time zero, 0% solvent B was used, followed by 30 min with 40% solvent B, 40 min with 75% solvent B, and 50 min with 100% solvent B.

**Analysis of total LMW and protein thiol amounts (DTNB method).** Cells were grown and treated with diamide as described above. To determine the amounts of LMW thiols, 50 ml of the culture was centrifuged and the pellet was resuspended in 600 µl extraction buffer. The suspension was incubated for 10 min at 60°C, and subsequently the cell debris was removed by centrifugation. Five hundred microliters of supernatant was mixed with 10 µl 100 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) in dimethyl sulfoxide. The pellet was again incubated with 600 µl extraction buffer, as described above, and 500 µl was added to the same tube used before. The A<sub>412</sub> was measured, and the LMW thiol content was calculated.

To determine the amount of protein thiols, the pellet harvested from 50 ml of culture was resuspended in 1,000 µl denaturing buffer, consisting of 8 M urea, 1% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 1 mM EDTA, 200 mM Tris-HCl (pH 8.0), and 10 µl of 100 mM DTNB was added directly. The suspension was incubated for 15 min and centrifuged. The supernatant was diluted, A<sub>412</sub> was measured, and the protein thiol content was calculated.

**Determination of the GSH/GSSG-ratio in the *B. subtilis gshF<sub>LM</sub>* strain.** Cells were cultured and treated with diamide as described above. Forty milliliters of the culture was centrifuged, and the pellet was resuspended in 400 µl extraction buffer. For GSSG determination, the buffer contained 3% scavenger reagent (GSH/GSSG assay kit from Oxford Biomedical Research). The suspension was incubated for 15 min at 60°C, and subsequently the cell debris was removed by centrifugation. Different dilutions were used for the assay according to the manufacturer's instructions.

**Protein isolation and alkylation of thiol groups.** Cells were harvested by centrifugation at 10,000 × g for 10 min at 4°C. The pellet was resuspended in denaturing buffer containing 100 mM iodoacetamide (IAM). Cells were disrupted by sonication, and the lysate was incubated in the dark for 20 min to allow alkylation of thiol groups. Proteins were precipitated in a fourfold volume of pure acetone. The resulting pellet was washed twice with acetone, dried in a SpeedVac, and resuspended in denaturing buffer without IAM.

**FALKO assay.** The two-dimensional (2D) gel-based FALKO assay was performed as described by Hochgräfe et al. (32). Proteins were isolated with alkylated thiol groups as described above. The protein concentration was measured according to Bradford et al. (9), and equal protein amounts of stressed and control samples were used. All reversible thiol modifications (disulfide bonds and

mixed disulfides) were reduced with Tris-(2-carboxyethyl)-phosphine (TCEP; Molecular Probes) and subsequently labeled with the fluorescence dye BODIPY FL C<sub>1</sub>-IA [*N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-yl)-methyl]-iodoacetamide; Molecular Probes] as described previously (32). The labeling reaction was stopped by centrifugation through a Bio-Rad Micro Bio-Spin 6 column. 2D polyacrylamide gel electrophoresis (PAGE), gel imaging, data analysis, and protein identification were performed as described elsewhere (32).

**Diagonal nonreducing/reducing SDS gel electrophoresis.** Proteins were isolated with alkylated thiol groups as described above. The concentration of protein was measured according to Bradford et al. (9), and equal protein amounts of all samples were applied. Samples were separated by SDS-PAGE under nonreducing conditions. The lanes of the SDS gel were cut and incubated in sample buffer (2% SDS, 62.5 mM Tris-HCl, pH 8.0) containing 50 mM dithiothreitol to reduce disulfide bonds. Subsequently, the lanes were incubated in sample buffer containing 100 mM IAM or 100  $\mu$ M BODIPY FL C<sub>1</sub>-IA, respectively. Each lane of the SDS gel was positioned vertically and separated again by SDS-PAGE. The resulting diagonal SDS gels were stained with SYPRO Ruby and scanned. Subsequently, proteins were stained with Coomassie brilliant blue as described previously (20) and scanned. Proteins that did not migrate on the diagonal were identified as described previously (20). For control purposes, SDS lanes were separated under reducing conditions in the first and second dimensions. Proteins that did not comigrate with the diagonal were omitted from further studies.

**[<sup>35</sup>S]cysteine monitoring of S thiolations.** The monitoring of S thiolations by [<sup>35</sup>S]cysteine was performed as described previously (33). In brief, protein synthesis was inhibited by addition of chloramphenicol for 30 min (*B. subtilis* *gshF<sub>Lm</sub>* strain) and fusidic acid for 60 min (*S. aureus*). The culture was supplemented with [<sup>35</sup>S]cysteine for 30 min, and subsequently cells were treated with diamide for 30 min or harvested as control samples. The proteins were isolated as described above. Equal protein amounts of all samples were applied to filter disks, and the radioactivity was measured in a scintillation counter. Aliquots of protein samples were reduced with TCEP prior to filter disk application to prove reducible S thiolations.

**Analyses of intracellular metabolites by GC-MS and LC-MS.** Analyses of intracellular metabolites by gas chromatography-mass spectrometry (GC-MS) were performed as described by Liebecke et al. (46). Briefly, samples were harvested by fast filtration, and subsequently the metabolism was quenched with an ethanol solution and liquid nitrogen. After cell disruption and metabolite extraction, samples were analyzed by GC-MS. Samples to detect possible disulfides and NADP(H) as well as ppGpp were analyzed by liquid chromatography (LC)-MS as described before (4, 18). An aminopropyl column (Phenomenex Luna NH<sub>2</sub>, 250 mm by 2 mm, 5  $\mu$ m) was used with solvent A (20 mM ammonium acetate, 20 mM ammonium hydroxide, 5% [vol/vol] ACN in water; pH 9.45) and solvent B (pure ACN). The flow rate was 0.2 ml  $\cdot$  min<sup>-1</sup>, with the following gradient: *t* = 0 min, 85% solvent B; *t* = 15 min, 0% solvent B; *t* = 38 min, 0% solvent B; *t* = 40 min, 85% solvent B; and *t* = 50 min, 85% solvent B. The mass spectrometer was adjusted as described by Donat et al. (18).

**GeLC-MS analysis.** Gel electrophoresis LC-MS (GeLC-MS) was carried out as described by Hochgräfe et al. (33). In brief, protein extracts of cells, stressed with diamide for 30 min, were isolated as described above. However, protein precipitation by acetone was omitted. The protein extracts were separated by nonreducing, one-dimensional SDS-PAGE. Gel lanes were sliced and each slice was digested with trypsin. The resulting peptide mixtures were separated by reversed-phase HPLC directly coupled to an LTQ Orbitrap mass spectrometer for subsequent tandem-MS (MS/MS) analysis. Extracts of *B. subtilis* *gshF<sub>Lm</sub>* cells were searched against database of *B. subtilis* 168 extracted from SubtiList (<http://genolist.pasteur.fr/SubtiList>). Extracts of *S. aureus* UAMS-1 cells were searched against a fusion protein database of all accessible *S. aureus* strains, including phage proteins and plasmid-encoded proteins.

## RESULTS

**Cysteine is used for S thiolations in *S. aureus* UAMS-1.** The cysteine synthase CysM has a crucial role in the regulation of cysteine biosynthesis and stress response of *S. aureus* to diamide (48, 59), suggesting an important role for cysteine or derivatives of cysteine. Recently, the LMW thiols cysteine, CoA, and BSH could be identified as abundant thiols in bacilli and *S. aureus* (52). We found S thiolations based on cysteine in *B. subtilis* in six proteins (33) and have extended these studies

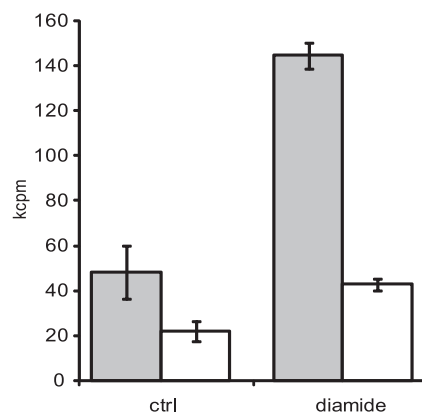


FIG. 1. Monitoring of S thiolations in *S. aureus* UAMS-1. In brief, protein synthesis was inhibited and subsequently [<sup>35</sup>S]Cys was supplied. Proteins of exponentially grown *S. aureus* cells (control [ctrl]) and those exposed to 2 mM diamide were isolated, and the bound radioactivity was measured (gray bars). To prove reversible S thiolations, parallel aliquots of the protein extracts were reduced prior to radioactivity measurement (white bars).

to analyze S thiolations in *S. aureus*. We used a [<sup>35</sup>S]cysteine-based assay described by Hochgräfe et al. (33). In brief, protein synthesis was inhibited by fusidic acid and [<sup>35</sup>S]cysteine was supplemented. Proteins were isolated from cells before and after exposure to diamide. The protein-associated radioactivity was measured before and after treatment with a reducing agent. A cysteine-free medium is required for the assay to ensure sufficient [<sup>35</sup>S]cysteine incorporation in S thiolations. *S. aureus* strains COL, UAMS-1, Newman, and NCTC 8325 and 6850 were examined for growth with different sulfur sources, including methionine, sulfate, thiosulfate, or thiocyanate. *S. aureus* UAMS-1 was the only strain that could assimilate thiosulfate and was therefore used for further investigations.

Exposure of cells to diamide led to 2.8-fold-higher protein-bound radioactivity compared to the untreated sample (Fig. 1). Reduction of the protein extract of diamide treated cells by TCEP caused a loss of protein-bound radioactivity of almost 70%, indicating that most of the [<sup>35</sup>S]cysteine was part of protein S thiolations. Protein extracts of *S. aureus* UAMS-1 exposed to diamide were subjected to GeLC-MS to identify LMW thiols responsible for S thiolations. Two proteins were identified that are modified by S cysteinylated: the acetoin reductase (SAR0129) is S cysteinylated at C-155 (see Fig. S1A in the supplemental material), and the hypothetical lipoprotein SAR0761 is S cysteinylated at C-133 (see Fig. S1B in the supplemental material). Results at a lower confidence level suggest S cysteinylations in a glycyl-tRNA synthetase (SAR1642) at C-344, a phosphoribosylaminoimidazole-succinocarboxamide synthase (SAR1040) at C-196, a pyruvate kinase (SAR1776) at C8, a triosephosphate isomerase (SAR0830) at C-40, and SarA (SAR0625) at C-9. Because all modified proteins were identified from the annotated genome of *S. aureus* MRSA252, we combined letters from this strain name into a prefix for our designations: “SAR.” Concluding these MS results, *S. aureus* uses S cysteinylated for protein thiol modification during disulfide stress.

**Analysis of inter- and intramolecular disulfide bonds in response to diamide stress.** Diamide stress leads to a massive increase of reversible oxidations of protein thiols in *B. subtilis*

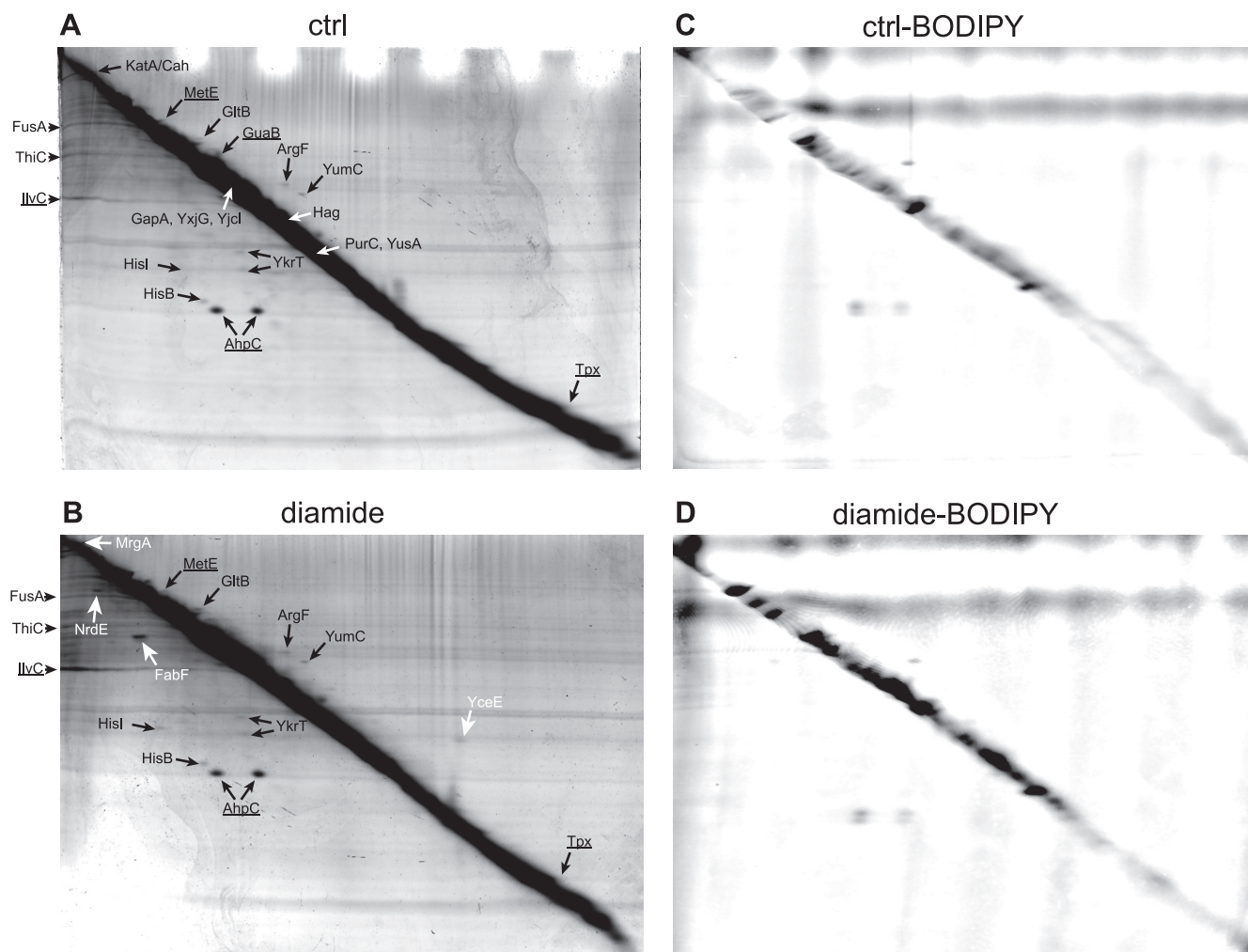


FIG. 2. Effect of diamide on intra- and intermolecular disulfide bond formation in *B. subtilis* 168. Gel images of protein extracts separated by diagonal gel electrophoresis are shown. Protein extracts of exponentially grown *B. subtilis* cells harvested before (A and C) or after (B and D) exposure to diamide were separated by nonreducing and subsequent reducing SDS-PAGE. Afterwards, proteins were stained with SYPRO Ruby (A and B). In a modified approach, protein thiols were labeled with BODIPY FL C<sub>1</sub>-1A instead of IAM (C and D) prior to the second separation and the fluorescence pattern was scanned. Underlined proteins were also identified as reversibly oxidized by Hochgräfe et al. (32). ctrl, control.

and *S. aureus* (32, 64). These modifications were identified as S cysteinylations in different proteins of *B. subtilis* (33) and *S. aureus* as shown here. We next analyzed the extent of newly formed inter- and intramolecular disulfide bonds in response to disulfide stress. For this purpose, the diagonal nonreducing/reducing SDS gel electrophoresis approach was used (57) for separation of untreated samples and those exposed to diamide.

Under control conditions, most proteins migrate along the diagonal, indicating their reduced state. The identification of proteins from randomly chosen gel pieces from the diagonal revealed proteins with and without cysteine residues in *B. subtilis* (Fig. 2A [e.g., KatA, Cah, Hag, GapA, and PurC]) and *S. aureus* (Fig. 3A [e.g., Asp23, RplD, and SAR0872]). This indicates that most proteins do not form intra- or intermolecular disulfide bonds under control conditions. Proteins that migrate below the diagonal include FusA, ThiC, IlvC, HisI, HisB, YkrT, YumC, and AhpC in *B. subtilis* (Fig. 2A) and AhpC, MetN-2, HutG, GlyQS, NrdE/Rir1, PurH, and a DeoD homologue in *S. aureus* (Fig. 3A). These proteins most likely

form intermolecular disulfide bonds even under control conditions. In *S. aureus* and *B. subtilis*, proteins above the diagonal were detected, indicating formation of intramolecular disulfide bonds (Fig. 2A and 3A). Six proteins of *B. subtilis* were identified above the diagonal as MetE, GltB, GuaB, ArgF, YumC, and Tpx. These contain at least two cysteines required for intramolecular disulfide bond generation. However, we have to consider that some proteins with intramolecular disulfide bonds might migrate close to the diagonal.

Diagonal gel electrophoresis of protein extracts from diamide-treated *B. subtilis* cells identified YceE with a newly formed intramolecular disulfide bond above the diagonal (Fig. 2B). Two proteins with newly formed intermolecular disulfide bonds were identified below the diagonal as the  $\beta$ -ketoacyl-acyl carrier protein synthase II FabF and the  $\alpha$  subunit of the oxygen-dependent ribonucleotide diphosphate reductase NrdE (Fig. 2B). FabF, NrdE, and YceE are not induced after exposure to diamide (45). Thus, we can exclude the appearance of these spots due to increased amounts of protein. Diamide also leads to an increase

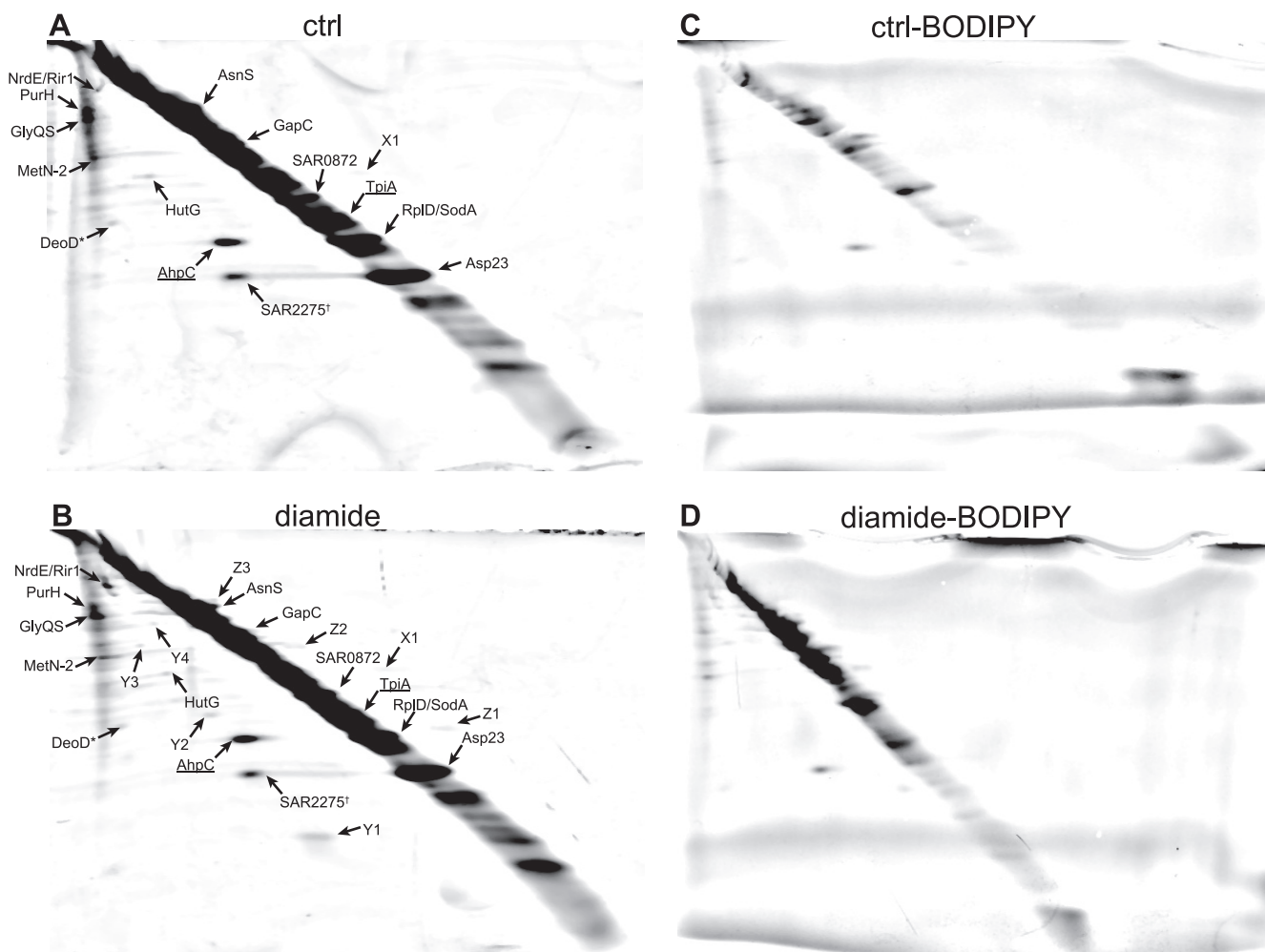


FIG. 3. Effect of diamide on intra- and intermolecular disulfide bond formation of *S. aureus* UAMS-1. Gel images of protein extracts separated by diagonal gel electrophoresis are shown. Protein extracts from exponentially grown *S. aureus* cells (A and C) and those exposed to diamide (B and D) were separated by nonreducing and subsequent reducing SDS-PAGE. Afterwards, proteins were stained with SYPRO Ruby (A and B). In a modified approach, protein thiols were labeled with BODIPY FL C<sub>1</sub>-IA instead of IAM (C and D) prior to the second separation and the fluorescence pattern was scanned. DeoD\*, purine-nucleoside phosphorylase, a DeoD homologue. SAR2275<sup>†</sup> contains 0 cysteines and was also detected at the same position in a reducing/reducing diagonal gel electrophoresis. Underlined proteins were also identified as reversibly oxidized by Wolf et al. (64). ctrl, control.

in disulfide bonds in *S. aureus*. We detected four proteins with newly formed intermolecular disulfide bonds (Fig. 3B [Y1 to Y4]) and three proteins with newly formed intramolecular disulfide bonds (Fig. 3B [Z1 to Z3]).

The diagonal gel electrophoresis approach does not visualize proteins which are S thiolated. To monitor all proteins with reversible thiol modifications, including S thiolations, we combined the diagonal gel electrophoresis approach with a thiol-specific fluorescent labeling. In brief, we separated the protein extracts with chemically IAM-alkylated thiol groups under nonreducing conditions. The excised gel lanes were reduced, and those proteins with reversible thiol oxidations were stained with the fluorescent dye BODIPY-FL C<sub>1</sub> IA. Proteins that are S thiolated migrate along the diagonal but are now fluorescently labeled. Many fluorescence signals were detected along the diagonal under control conditions (Fig. 2C and 3C), which increased after diamide stress (Fig. 2D and 3D) in *B. subtilis*

and in *S. aureus*. Comparison of the images from the original approach and this combined approach did not result in the detection of additional protein spots apart from the diagonal. These findings further support the formation of S thiolations in *B. subtilis* and *S. aureus* after diamide stress.

**Diamide leads to decreased amounts of the LMW thiol cysteine and other changes in intracellular metabolites of *B. subtilis*.** We investigated the global intracellular metabolite pool—the metabolome—to follow changes in the level of LMW thiols and other compounds after disulfide stress in response to diamide.

The cysteine pool decreased after exposure to diamide by about 15-fold (Fig. 4A). However, we did not detect formation of cystine, the cysteine disulfide, or other disulfides at the given sample points. We also monitored cysteine precursors, *O*-acetylserine, serine, and methionine. Methionine levels decreased about fourfold during disulfide stress (Fig. 4A). *O*-Acetylserine levels increased up to 20-fold, whereas serine levels did not

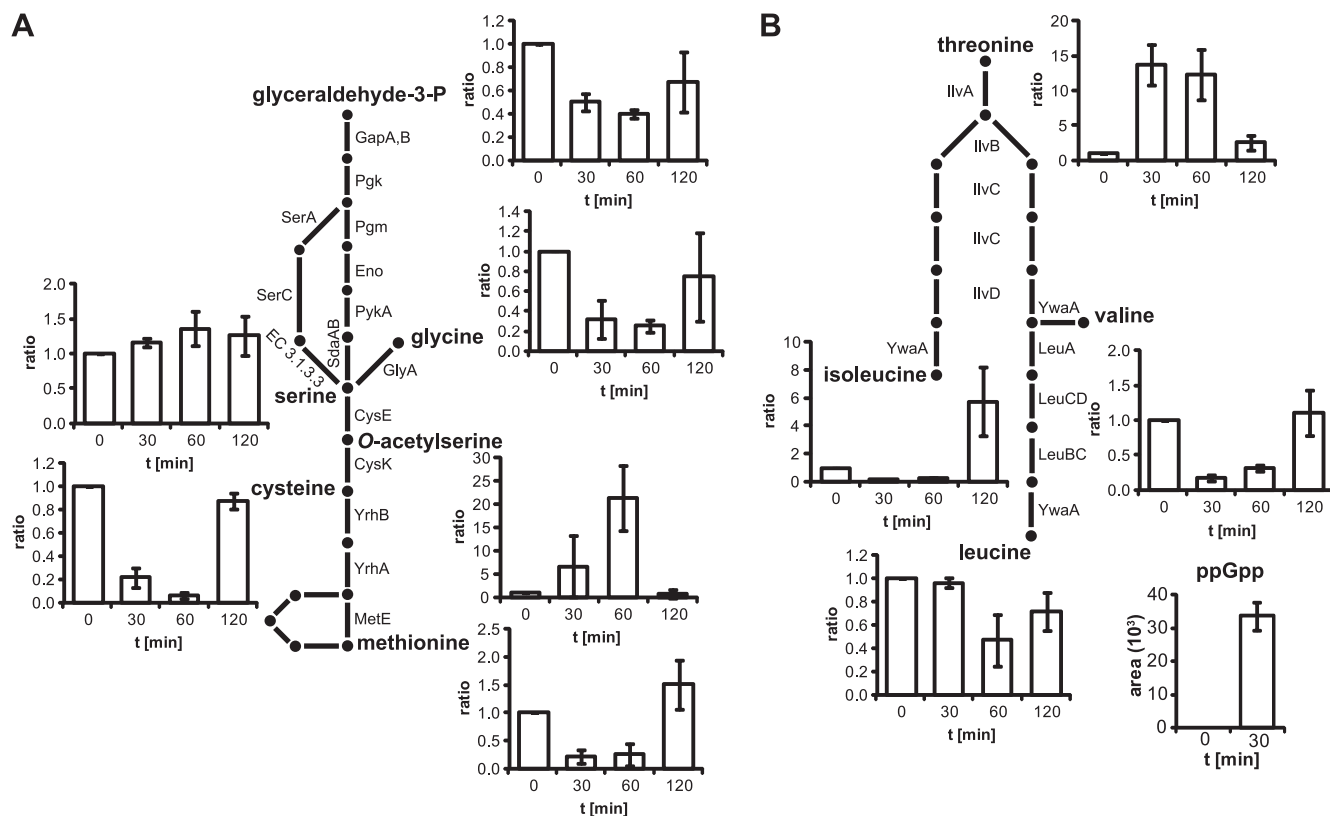


FIG. 4. Global metabolomic changes in the cellular amounts of different metabolites of *B. subtilis* after exposure to diamide. Cytoplasmic extracts of exponentially grown *B. subtilis* cells (0 min) and those exposed to 1 mM diamide (30, 60, and 120 min) were measured by GC-MS or LC-MS as described in Materials and Methods. Control values are set to 1, and ratios of the diamide samples are shown at their sample points, except for ppGpp. The pathways of biosynthesis of cysteine (A) and branched-chain amino acids (B) are depicted with metabolites as dots and enzyme reactions as lines.

change significantly (Fig. 4A). The pool of glycine, a serine precursor, was depleted up to 75% in diamide-treated cells (Fig. 4A). Additionally, serine can be synthesized via pyruvate or the phospho-serine-pathway (56). Both use glyceraldehyde-3-phosphate as a precursor. The concentration of glyceraldehyde-3-phosphate decreased up to 50% during disulfide stress (Fig. 4A). The levels of cysteine, methionine, *O*-acetylserine, glycine, and glyceraldehyde-3-phosphate were restored to normal 120 min after exposure to diamide. This restoration of the metabolite pool occurred in parallel to the restoration of growth (45).

The metabolomic data further showed increased amounts of threonine (14-fold) after exposure to diamide (Fig. 4B). Threonine is a precursor for the synthesis of the branched-chain amino acids leucine, isoleucine, and valine. Diamide leads to a decrease in the levels of valine (5.5-fold) and isoleucine (4-fold), while the amount of leucine decreased later, and not as markedly (Fig. 4B). Additionally, synthesis of ppGpp, the alarmon of the stringent response, could be observed during disulfide stress (Fig. 4B).

These observations revealed significant modifications in the cysteine and branched-chain amino acid biosynthesis pathways in response to diamide. The drastic depletion of cysteine and its precursors supports the idea of S thiolation of protein thiols after exposure to diamide.

**Expression of the *L. monocytogenes gshF* gene in *B. subtilis* enables GSH production.** The results described above suggest that *B. subtilis* and *S. aureus* possess a thiol redox buffer based on cysteine. However, neither of these bacteria is able to produce GSH. The *gshF* gene of *Listeria monocytogenes*, a close relative of *B. subtilis*, encodes a multidomain GSH synthase (29). By heterologous expression of *L. monocytogenes gshF* in a *B. subtilis* gene, we engineered the *B. subtilis gshF<sub>Lm</sub>* strain.

To determine expression of GshF in the *B. subtilis gshF<sub>Lm</sub>* strain, protein extracts from IPTG-induced and noninduced cells were separated by 2D PAGE. The GshF (Imo2770) protein could be identified in the gel of the IPTG-induced *B. subtilis gshF<sub>Lm</sub>* strain (Fig. 5B). Growth of the *B. subtilis gshF<sub>Lm</sub>* strain was not inhibited by IPTG addition (Fig. 5A). To assess production of GSH in the *B. subtilis gshF<sub>Lm</sub>* strain, whole-cell lysates were labeled with the thiol-reactive compound mBBr and separated by HPLC. In parallel, control samples were incubated with NEM prior to exposure to mBBr. NEM binds to thiols and thus inhibits the subsequent binding of mBBr. Hence, only peaks in the mBBr sample account for thiols that are not present in the NEM sample. The IPTG-induced *B. subtilis gshF<sub>Lm</sub>* strain contained GSH at a concentration of 2.63  $\mu\text{mol/g}$  DCW and  $\gamma$ -glutamylcysteine ( $\gamma$ -GC), the immediate precursor of GSH, at a concentration of 0.74

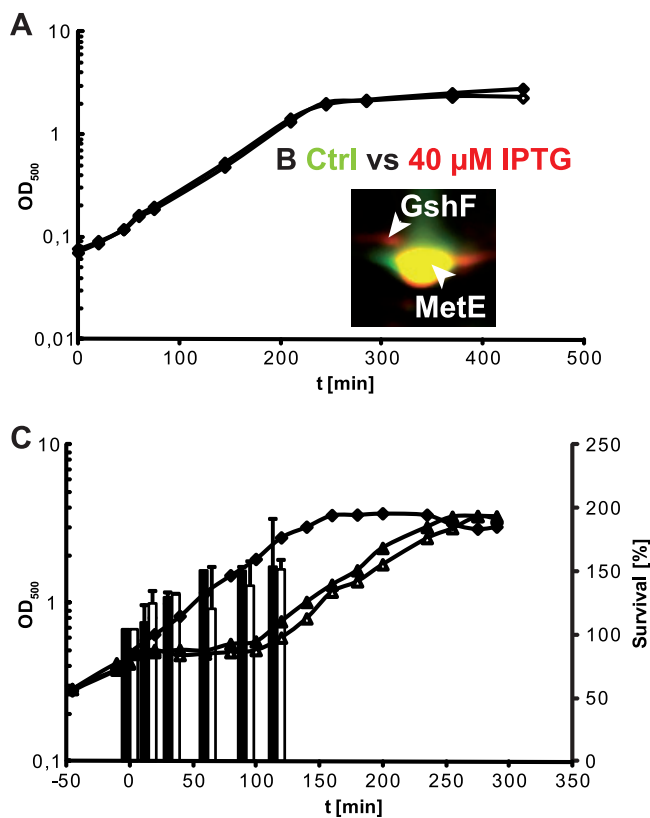


FIG. 5. Growth curves and survival ratios of *B. subtilis gshF<sub>Lm</sub>* strain cells exposed to diamide. (A) Growth curve of *B. subtilis gshF<sub>Lm</sub>* strain without (empty squares) and with (filled squares) 40  $\mu$ M IPTG. Ctrl, control. (B) The MetE region is shown as section of the dual-channel images of protein extracts stained with SYPRO Ruby from the noninduced (green) and IPTG-induced *B. subtilis gshF<sub>Lm</sub>* strain (red). (C) Growth curves (lines) and survival ratios (bars) of the *B. subtilis gshF<sub>Lm</sub>* strain exposed to 1 mM diamide with (filled triangles and bars) or without (empty triangles and bars) 40  $\mu$ M IPTG. As a control, *B. subtilis gshF<sub>Lm</sub>* strain cells without IPTG were grown (filled squares).

$\mu$ mol/g DCW (Fig. 6B and F). GSH and  $\gamma$ -GC were hardly detectable in the noninduced *B. subtilis gshF<sub>Lm</sub>* strain cultures. Cysteine and CoA were present under noninducing conditions at concentrations of 0.25 and 0.87  $\mu$ mol/g DCW, respectively (Fig. 6D and F). In response to GSH production, cysteine (0.31  $\mu$ mol/g DCW) and CoA (0.8  $\mu$ mol/g DCW) were detected at similar concentrations (Fig. 6B and F). Analyses of the whole LMW thiol amount by the DTNB method showed that it exceeded the sum of thiols analyzed by this HPLC method (Fig. 6F and G). Not all thiols could be quantified by the HPLC method, and differences in the preparation methods might explain this observation. We found whole LMW thiol amounts of 5.42  $\mu$ mol/g DCW in the GSH-free *B. subtilis gshF<sub>Lm</sub>* strain and 13.39  $\mu$ mol/g DCW in the GSH-producing *B. subtilis gshF<sub>Lm</sub>* strain (Fig. 6G).

**GSH production in the *B. subtilis gshF<sub>Lm</sub>* strain enables S glutathionylation but provides no protection against disulfide and oxidative stress.** We were interested in whether GSH produced by the *B. subtilis gshF<sub>Lm</sub>* strain contributes to the thiol redox buffer. Phenotypical experiments revealed that GSH production had no discernible effect on growth or survival under

thiol-specific stress conditions (Fig. 5C). Additionally, we analyzed this strain for oxidative stress resistance induced by H<sub>2</sub>O<sub>2</sub> and the superoxide-generating agent methyl viologen. During peroxide stress, no significant changes could be detected in growth or survival due to production of GSH (see Fig. S2A in the supplemental material). However, during superoxide stress, cells without GSH recovered slightly faster than cells with GSH (see Fig. S2B in the supplemental material).

Analyses of the whole LMW thiol amount by the DTNB method revealed no significant difference between noninduced (1.33  $\mu$ mol/g DCW) and induced (1.91  $\mu$ mol/g DCW) *B. subtilis gshF<sub>Lm</sub>* strain cells after exposure to diamide (Fig. 6G). This suggests a strong oxidation of GSH, which could be verified by measuring the GSH/GSSG ratio (Fig. 6I). In the exponential growth phase, GSH was present at 8.67  $\mu$ mol/g DCW and GSSG at 0.03  $\mu$ mol/g DCW, giving a ratio of 267.67:1. Under diamide stress, the concentration of GSH was 1.95  $\mu$ mol/g DCW and that of GSSG was 2.61  $\mu$ mol/g DCW, resulting in a ratio of 1:1.34.

Next, we analyzed the thiol redox proteome of the *B. subtilis gshF<sub>Lm</sub>* strain using the FALKO assay, the diagonal gel electrophoresis methodology, and the S thiolation assay based on [<sup>35</sup>S]cysteine to detect differences in reversible thiol modifications of proteins. Proteins of GSH-producing and GSH-free *B. subtilis gshF<sub>Lm</sub>* strain cells were analyzed before and after exposure to diamide. Analyses of reversible thiol modifications using the FALKO assay showed no significant differences based on GSH production, either under control conditions or in response to diamide stress (see Fig. S3 in the supplemental material). Furthermore, comparison of the diagonal electrophoretic patterns from noninduced and GSH-producing, *B. subtilis gshF<sub>Lm</sub>* strain cells did not show significant changes in the formation of disulfide bonds before and after diamide stress (see Fig. S4 in the supplemental material). This is supported by quantitative analyses of protein thiols in the *B. subtilis gshF<sub>Lm</sub>* strain (Fig. 6H). We measured 12.89  $\mu$ mol/g DCW in GSH-free cells and 12.98  $\mu$ mol/g DCW in GSH-producing cells under control conditions. Diamide led to a loss of 60% of reduced protein thiols in GSH-free (5.26  $\mu$ mol/g DCW) and GSH-producing (5.17  $\mu$ mol/g DCW) cells. Thus, GSH production did not affect the level of reduced protein thiols. Remarkably, [<sup>35</sup>S]cysteine incorporation of GSH-producing *B. subtilis gshF<sub>Lm</sub>* strain cells decreased 2.8-fold compared to the level in the noninduced strain (Fig. 7A and B, left columns). This difference might result from a higher pool of LMW thiols in the GSH-producing strain (Fig. 6F and H). Exposure to diamide resulted in a comparable increase of [<sup>35</sup>S]cysteine incorporation in both GSH-producing (5.3-fold) and nonproducing (5.9-fold) cultures. Reduction of samples treated with diamide by TCEP resulted in an 80% loss of radioactivity in the GSH-free sample and 65% loss in the GSH-containing sample (Fig. 7A and B). The marked loss of radioactivity following protein reduction indicates S thiolations.

To assess whether S glutathionylation of proteins occurs, and if in such instances the proteins were previously known to be S thiolated, we analyzed the protein extracts of diamide-treated GSH-producing *B. subtilis gshF<sub>Lm</sub>* strain cells by GeLC-MS. We found S glutathionylation in YwaA at C-104 (see Fig. S5 in the supplemental material). YwaA was previously shown to be S cysteinylated in *B. subtilis* wild-type cells

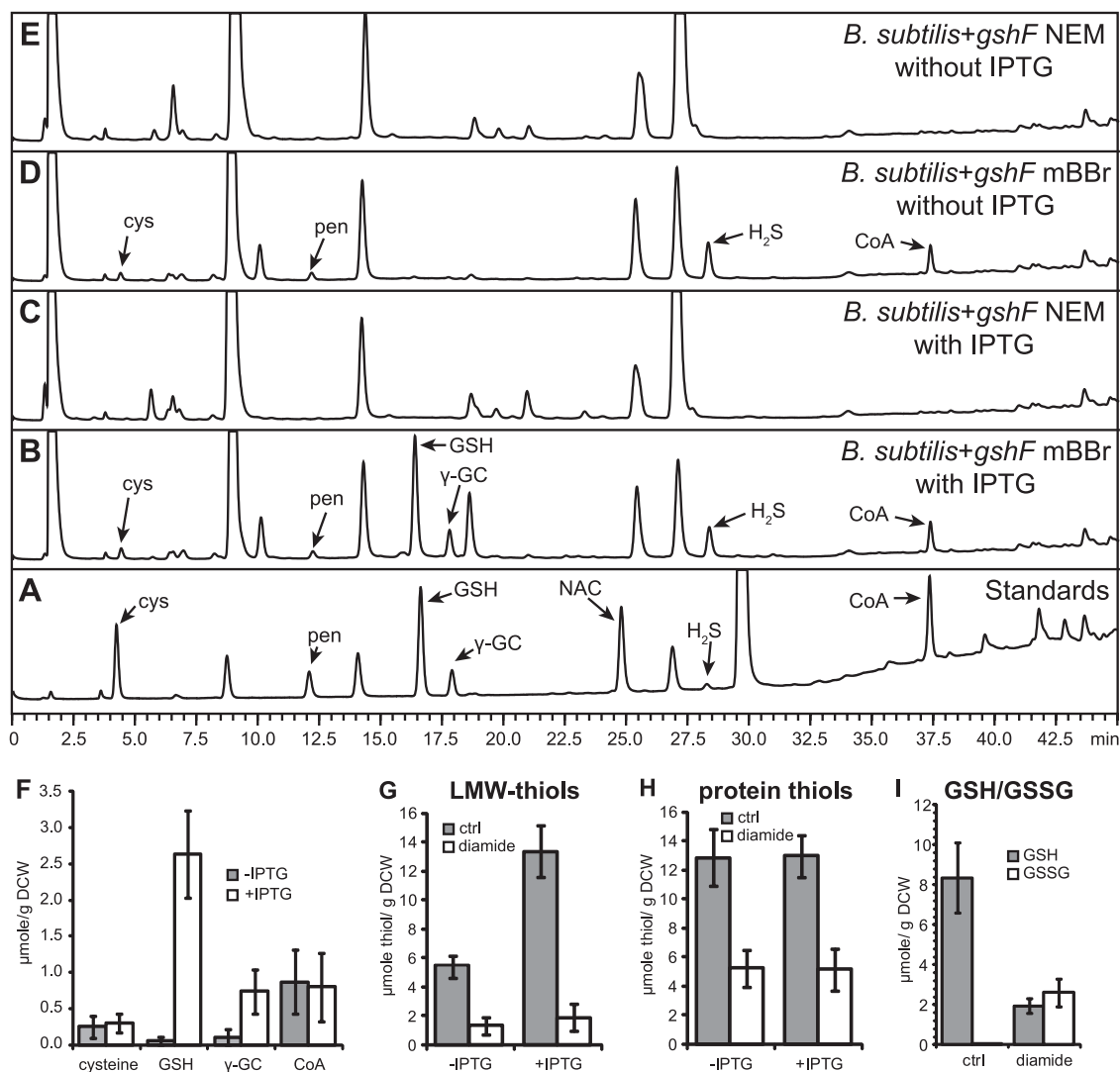


FIG. 6. Analysis of LMW and protein thiols of the *B. subtilis gshF<sub>Lm</sub>* strain. LMW thiols were separated as their bimeane derivatives of the IPTG-induced (B) or noninduced (D) *B. subtilis gshF<sub>Lm</sub>* strain cells. (The *gshF<sub>Lm</sub>* strain is indicated as “+*gshF*” on the figure.) For control reasons, NEM derivatives of equally treated cells were separated (C and E). Peaks were compared with a chromatogram of a standard mixture of thiols (A) containing cysteine (cys), penicillamine (pen [internal standard in all samples]), GSH,  $\gamma$ -glutamyl-cysteine ( $\gamma$ -GC), *N*-acetylcysteine (NAC), sulfide (H<sub>2</sub>S) and CoA. The concentrations of different thiols of the samples treated with mBBR were calculated using dilutions of the standard mixture (F) of noninduced (gray bars) and induced (white bars) cultures. LMW thiols (G) were extracted from noninduced (–IPTG) and induced (+IPTG) *B. subtilis gshF<sub>Lm</sub>* strain cells before (gray bars) and after (white bars) exposure to diamide. LMW thiols were quantified by DTNB. The remaining cell pellets of noninduced (–IPTG) and induced (+IPTG) *B. subtilis gshF<sub>Lm</sub>* strain cells before (gray bars) and after (white bars) exposure to diamide were resuspended in denaturing buffer, and (H) protein thiols were quantified using DTNB. (I) The ratio of GSH (gray bars) and GSSG (white bars) was analyzed in induced *B. subtilis gshF<sub>Lm</sub>* strain cells under control (ctrl) and disulfide stress (diamide) conditions.

(33). Preliminary data show further examples of S glutathionylations in YjcI (C-128), Tsf (C-239), PurB (C-199), YjdL (C-177), and MetE (C-719). Although cysteine is present in GSH-producing *B. subtilis gshF<sub>Lm</sub>* strain cells, we did not find any S cysteinylations.

## DISCUSSION

Lithgow et al. showed that inactivation of the cysteine synthase CysM in *S. aureus* SH1000 results in decreased resistance against oxidative stress (48). Furthermore, they detected a depleted cysteine pool in the *cysM*-lacking mutant. The dimin-

ished oxidative stress resistance is probably linked to this depletion. Here we provide evidence that cysteine participates in the formation of S thiolations in *S. aureus*, as revealed by the [<sup>35</sup>S]cysteine assay and the GeLC-MS approach. Thus, it is likely that cysteine functions as thiol redox buffer in *S. aureus* and *B. subtilis*. BSH, another abundant cysteine-derived thiol in both organisms, is most likely also a key player in this buffer (52). CoA may also contribute to this buffer, which is an abundant thiol in *S. aureus* (49). Moreover, *S. aureus* possesses a CoA-disulfide reductase which strengthens a possible role of CoA as thiol redox-buffer (14, 15).

Using the diagonal gel electrophoresis, we confirmed that

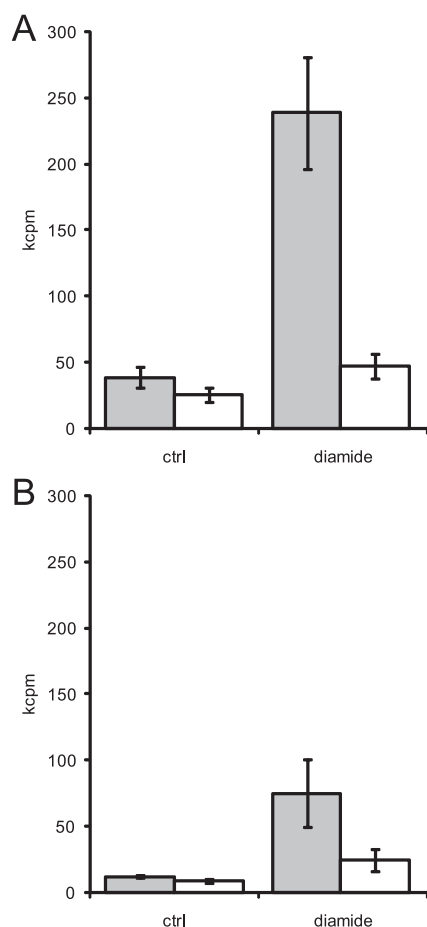


FIG. 7. Monitoring of S thiolations in the *B. subtilis* *gshF<sub>Lm</sub>* strain. In brief, protein synthesis was inhibited and subsequently [<sup>35</sup>S]Cys was supplied. Proteins of exponentially grown *B. subtilis* *gshF<sub>Lm</sub>* strain cells (control [ctrl]) and those exposed to 1 mM diamide (diamide) without (A) or with (B) 40 μM IPTG were isolated, and the bound radioactivity was measured (gray bars). To prove reversible S thiolations, parallel aliquots of the protein extracts were reduced prior to radioactivity measurement (white bars).

redox-active enzymes, such as AhpC and Tpx, form disulfide bonds during their catalytic cycle. AhpC forms homomeric or heteromeric intermolecular disulfide bonds with its redox partner protein, AhpF (38). In contrast, the thiol-dependent peroxidase Tpx forms intramolecular disulfide bonds (5).

NrdE, a subunit of the class Ib ribonucleotide reductase, seems to undergo increased intermolecular disulfide bond formation with a redoxin in response to diamide (22, 31). FabF, the β-ketoacyl carrier protein synthase II, forms an intermolecular disulfide bond under disulfide stress. FabF in *Streptococcus pneumoniae* possesses an active-site thiolate and occurs in *E. coli* as a homodimer (25, 36). These observations suggest that active-site cysteines of FabF lead to intermolecular, homodimeric disulfide bond formation under diamide stress due to their sterical proximity. FabHB, the β-ketoacyl carrier protein synthase III, catalyzes the same reactions as FabF (63) and is induced in diamide-treated *B. subtilis* cells (45). These findings suggest a reduced activity of FabF which could be compensated for by FabHB induction.

In general, the diagonal gel electrophoresis approach of diamide-treated *B. subtilis* and *S. aureus* cells supports the idea that the majority of disulfide bonds (32, 64) are mixed disulfides of protein thiols with LMW thiols rather than inter- and intramolecular disulfide bonds. However, we have to consider that some cysteine-containing proteins may be below the detection level of this assay or could have cysteine residues that are not solvent exposed and not accessible for thiol modifications.

The metabolome data revealed depletion of cysteine after exposure to diamide. Under these thiol-specific stress conditions, cysteine biosynthesis is induced by the derepression of the CymR regulon (19, 45). We found an increase of *O*-acetylserine, which leads to dissociation of the CymR-CysK complex and derepression of the CymR regulon (61). The concentration of serine, the precursor of *O*-acetylserine, did not change significantly. However, the concentration of glycine, which is a precursor of serine, is reduced under disulfide stress and the synthesis of the glycyl-tRNA synthetase is also decreased (45). These results might indicate a fortified provision of glycine for serine synthesis.

The CymR-controlled MccA (YrhA) and MccB (YrhB) proteins participate in the conversion of methionine to cysteine and are induced under disulfide stress (37, 45). MetE, the cobalamin-independent methionine synthase, is S cysteinylated under diamide stress and less active in the S-thiolated state in *E. coli* (33, 35). We observed that the methionine pool was fourfold decreased in diamide-treated cells. These findings indicate an increased conversion of methionine to cysteine and simultaneously a decreased synthesis of methionine.

Free transition metals such as iron, copper, and zinc can be coordinated by cysteine (50). Disulfide bond formation in Zn-binding proteins such as RsrA and Hsp33 releases Zn (44, 54). However, it seems unlikely that diamide leads to depletion of cysteine caused by complexation of released metal ions. We suggest that the cysteine pool is drastically depleted due to formation of S thiolation based on cysteine or related compounds. Thus, cysteine biosynthesis is induced from different precursors after exposure to diamide in *B. subtilis*.

After exposure to diamide, the cellular amount of LMW thiols decreased from 5.42 to 1.33 μmol/g DCW (4.09 μmol/g DCW = 75%) and that of protein thiols decreased from 12.89 to 5.26 μmol/g DCW (7.63 μmol/g DCW = 60%). Using GC or LC-MS analyses, no LMW disulfides were detected. It has to be considered that formation of an intra- and intermolecular disulfide bond needs two protein thiols. Taken together, this allows an approximation that about 70% diamide-provoked disulfide bonds consist of S thiolations (see Fig. S6 in the supplemental material). Hansen et al. exposed eukaryotic HEK and HeLa cells to diamide and detected a 300-fold increase in S glutathionylations in these cells, thereby using a third of the GSH pool (30). Concomitantly, oxidation to GSSG increased fivefold, using 50% of the GSH pool. These results show that a large fraction of LMW thiols is used for S thiolations under disulfide stress.

Furthermore, we detected under disulfide stress an increase in the concentration of threonine, whereas the concentrations of the threonine-derived amino acids, leucine, isoleucine, and valine, were decreased. The ketol-acid reductoisomerase IlvC

and the branched-chain amino acid aminotransferase YwaA are involved in the conversion of threonine to leucine, isoleucine, and valine. Both proteins undergo reversible thiol modifications after exposure to diamide (32). It might be possible that diamide modifies the functionality of YwaA and IlvC and correspondingly affects synthesis of the branched-chain amino acids. IlvC uses NADPH in its reaction, and oxidoreductases use NADPH as well. Thus, the competition for NADPH could be responsible for the decrease of the branched-chain amino acids. However, analysis of NADP<sup>+</sup> and NADPH revealed no significant difference in the redox ratio (see Fig. S7 in the supplemental material). This indicates that the fortified use of NADPH by oxidoreductases does not affect the pool of NADPH and thereby the function of other NADPH-dependent enzymes, such as IlvC.

The depletion of different amino acids can activate the stringent response in *B. subtilis* (62). The alarmone of this adaptation process, ppGpp, was detected during diamide stress, but not during exponential growth. This confirms transcriptome data that diamide triggers the stringent response (45).

Based on the above studies, we were interested whether in *B. subtilis* GSH could support cysteine in the maintenance of the thiol redox-buffer. To this end, we analyzed the global thiol oxidation state and S thiolations in a GSH-producing *B. subtilis* strain. Different approaches used for assaying reversible thiol oxidations failed to reveal any effect of GSH in cells subjected to disulfide stress. An S glutathionylation in YwaA was detected, but no S thiolations by other compounds were identified. This may be related to the increased GSH content in the GSH-producing strain, which leads to a drastic change in the ratio of GSH to other LMW thiols.

Fu et al. (24) introduced GSH production into *Lactococcus lactis* subsp. *cremoris* NZ9000. They found that GSH production led to impaired growth due to increased cysteine utilization. Exposure to oxidants (H<sub>2</sub>O<sub>2</sub> and the superoxide-generating agent menadione) did not affect the survival of exponentially growing cells with GSH compared to cells without GSH. However, during the transition phase, cells benefit from GSH production: the rate of survival was increased after exposure to oxidants. With the artificial introduction of GSH synthesis, the GSH system was complete in *L. lactis* (24), including GSH reductase, GSH peroxidase, glutaredoxin, and GSH. In contrast to *L. lactis*, *B. subtilis* lacks the GSH reductase and the GSH peroxidase, suggesting that GSH can be oxidized in *B. subtilis* to GSSG but not rereduced to GSH. We cannot exclude that the thioredoxin system is capable of this redox-reaction in *B. subtilis*.

In summary, different approaches were used to analyze the nature of reversible thiol oxidations in *B. subtilis* and *S. aureus* in response to diamide stress. S thiolation is the major mechanism used to protect cellular protein thiols in both bacteria after exposure to diamide. The decreased amount of the LMW thiol cysteine supports its major role as a thiol redox buffer. However, cysteine is also used for the BSH synthesis, and identified S cysteinylations may be degradation products of previous S bacillithiolations. Thus, future studies will be directed at identifying the precise thiol redox buffer which is used for S thiolations in *B. subtilis* and *S. aureus*.

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## REFERENCES

1. Anagnostopoulos, C., and J. Spizizen. 1961. Requirements for transformation in *Bacillus subtilis*. *J. Bacteriol.* **81**:741–746.
2. Antelmann, H., M. Hecker, and P. Zuber. 2008. Proteomic signatures uncover thiol-specific electrophile resistance mechanisms in *Bacillus subtilis*. *Expert Rev. Proteomics* **5**:77–90.
3. Arnér, E. S., and A. Holmgren. 2000. Physiological functions of thioredoxin and thioredoxin reductase. *Eur. J. Biochem.* **267**:6102–6109.
4. Bajad, S. U., W. Lu, E. H. Kimball, J. Yuan, C. Peterson, and J. D. Rabinowitz. 2006. Separation and quantitation of water soluble cellular metabolites by hydrophilic interaction chromatography-tandem mass spectrometry. *J. Chromatogr. A* **1125**:76–88.
5. Baker, L. M., and L. B. Poole. 2003. Catalytic mechanism of thiol peroxidase from *Escherichia coli*. *J. Biol. Chem.* **278**:9203–9211.
6. Bardwell, J. C. 1994. Building bridges: disulfide bond formation in the cell. *Mol. Microbiol.* **14**:199–205.
7. Barford, D. 2004. The role of cysteine residues as redox-sensitive regulatory switches. *Curr. Opin. Struct. Biol.* **14**:679–686.
8. Biswas, S., A. S. Chida, and I. Rahman. 2006. Redox modifications of protein-thiols: emerging roles in cell signaling. *Biochem. Pharmacol.* **71**:551–564.
9. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254.
10. Carmel-Harel, O., and G. Storz. 2000. Roles of the glutathione- and thioredoxin-dependent reduction systems in the *Escherichia coli* and *Saccharomyces cerevisiae* responses to oxidative stress. *Annu. Rev. Microbiol.* **54**:439–461.
11. Chen, P. R., S. Nishida, C. B. Poor, A. Cheng, T. Bae, L. Kuechenmeister, P. M. Dunman, D. Missiakas, and C. He. 2009. A new oxidative sensing and regulation pathway mediated by the MgrA homologue SarZ in *Staphylococcus aureus*. *Mol. Microbiol.* **71**:198–211.
12. Choi, S.-Y., D. Reyes, M. Leelakriangsak, and P. Zuber. 2006. The global regulator Spx functions in the control of organosulfur metabolism in *Bacillus subtilis*. *J. Bacteriol.* **188**:5741–5751.
13. Cumming, R. C., N. L. Andon, P. A. Haynes, M. Park, W. H. Fischer, and D. Schubert. 2004. Protein disulfide bond formation in the cytoplasm during oxidative stress. *J. Biol. Chem.* **279**:21749–21758.
14. delCardayré, S. B., and J. E. Davies. 1998. *Staphylococcus aureus* coenzyme A disulfide reductase, a new subfamily of pyridine nucleotide-disulfide oxidoreductase. *J. Biol. Chem.* **273**:5752–5757.
15. delCardayré, S. B., K. P. Stock, G. L. Newton, R. C. Fahey, and J. E. Davies. 1998. Coenzyme A disulfide reductase, the primary low molecular weight disulfide reductase from *Staphylococcus aureus*. *J. Biol. Chem.* **273**:5744–5751.
16. Demple, B. 2002. Signal transduction by nitric oxide in cellular stress responses. *Mol. Cell. Biochem.* **234–235**:11–18.
17. Di Simplicio, P., F. Franconi, S. Frosali, and D. Di Giuseppe. 2003. Thiolation and nitrosation of cysteines in biological fluids and cells. *Amino Acids* **25**:323–339.
18. Donat, S., K. Streker, T. Schirmeister, S. Rakette, T. Stehle, M. Liebeke, M. Lalk, and K. Ohlsen. 2009. Transcriptome and functional analysis of the eukaryotic-type serine/threonine kinase PknB in *Staphylococcus aureus*. *J. Bacteriol.* **191**:4056–4069.
19. Even, S., P. Burguière, S. Auger, O. Soutourina, A. Danchin, and I. Martin-Verstraete. 2006. Global control of cysteine metabolism by CymR in *Bacillus subtilis*. *J. Bacteriol.* **188**:2184–2197.
20. Eymann, C., A. Dreisbach, D. Albrecht, J. Bernhardt, D. Becher, S. Gentner, L. T. Tam, K. Büttner, G. Buurman, C. Scharf, S. Venz, U. Völker, and M. Hecker. 2004. A comprehensive proteome map of growing *Bacillus subtilis* cells. *Proteomics* **4**:2849–2876.
21. Fahey, R. C., and G. L. Newton. 1987. Determination of low-molecular-

- weight thiols using monobromobimane fluorescent labeling and high-performance liquid chromatography. *Methods Enzymol.* **143**:85–96.
22. Fontecave, M. 1998. Ribonucleotide reductases and radical reactions. *Cell. Mol. Life Sci.* **54**:684–695.
  23. Fontecave, M., and S. Ollagnier-de-Choudens. 2008. Iron-sulfur cluster biosynthesis in bacteria: mechanisms of cluster assembly and transfer. *Arch. Biochem. Biophys.* **474**:226–237.
  24. Fu, R. Y., R. S. Bongers, I. I. van Swam, J. Chen, D. Molenaar, M. Kleerebezem, J. Hugenholtz, and Y. Li. 2006. Introducing glutathione biosynthetic capability into *Lactococcus lactis* subsp. *cremoris* NZ9000 improves the oxidative-stress resistance of the host. *Metab. Eng.* **8**:662–671.
  25. Garwin, J. L., A. L. Klages, and J. E. Cronan, Jr. 1980. Structural, enzymatic, and genetic studies of  $\beta$ -ketoacyl-acyl carrier protein synthases I and II of *Escherichia coli*. *J. Biol. Chem.* **255**:11949–11956.
  26. Gertz, S., S. Engelmann, R. Schmid, K. Ohlsen, J. Hacker, and M. Hecker. 1999. Regulation of  $\sigma^P$ -dependent transcription of *sigB* and *asp23* in two different *Staphylococcus aureus* strains. *Mol. Gen. Genet.* **261**:558–566.
  27. Ghezzi, P., B. Romines, M. Fratelli, I. Eberini, E. Gianazza, S. Casagrande, T. Laragione, M. Mengozzi, L.A. Herzenberg, and L. A. Herzenberg. 2002. Protein glutathionylation: coupling and uncoupling of glutathione to protein thiol groups in lymphocytes under oxidative stress and HIV infection. *Mol. Immunol.* **38**:773–780.
  28. Gillaspay, A. F., S. G. Hickmon, R. A. Skinner, J. R. Thomas, C. L. Nelson, and M. S. Smeltzer. 1995. Role of the accessory gene regulator (*agr*) in pathogenesis of staphylococcal osteomyelitis. *Infect. Immun.* **63**:3373–3380.
  29. Gopal, S., I. Borovok, A. Ofer, M. Yanku, G. Cohen, W. Goebel, J. Krefz, and Y. Aharonowitz. 2005. A multidomain fusion protein in *Listeria monocytogenes* catalyzes the two primary activities for glutathione biosynthesis. *J. Bacteriol.* **187**:3839–3847.
  30. Hansen, R. E., D. Roth, and J. R. Winther. 2009. Quantifying the global cellular thiol-disulfide status. *Proc. Natl. Acad. Sci. USA* **106**:422–427.
  31. Härtig, E., A. Hartmann, M. Schätzle, A. M. Albertini, and D. Jahn. 2006. The *Bacillus subtilis* *rdEF* genes, encoding a class Ib ribonucleotide reductase, are essential for aerobic and anaerobic growth. *Appl. Environ. Microbiol.* **72**:5260–5265.
  32. Hochgräfe, F., J. Mostertz, D. Albrecht, and M. Hecker. 2005. Fluorescence thiol modification assay: oxidatively modified proteins in *Bacillus subtilis*. *Mol. Microbiol.* **58**:409–425.
  33. Hochgräfe, F., J. Mostertz, D. C. Pöther, D. Becher, J. D. Helmann, and M. Hecker. 2007. S-cysteinylation is a general mechanism for thiol protection of *Bacillus subtilis* proteins after oxidative stress. *J. Biol. Chem.* **282**:25981–25985.
  34. Holmgren, A., C. Johansson, C. Berndt, M. E. Lönn, C. Hudemann, and C. H. Lillig. 2005. Thiol redox control via thioredoxin and glutaredoxin systems. *Biochem. Soc. Trans.* **33**:1375–1377.
  35. Hondorp, E. R., and R. G. Matthews. 2009. Oxidation of cysteine 645 of cobalamin-independent methionine synthase causes a methionine limitation in *Escherichia coli*. *J. Bacteriol.* **191**:3407–3410.
  36. Huang, W., J. Jia, P. Edwards, K. Dehesh, G. Schneider, and Y. Lindqvist. 1998. Crystal structure of  $\beta$ -ketoacyl-acyl carrier protein synthase II from *E. coli* reveals the molecular architecture of condensing enzymes. *EMBO J.* **17**:1183–1191.
  37. Hullo, M.-F., S. Auger, O. Soutourina, O. Barzu, M. Yvon, A. Danchin, and I. Martin-Verstraete. 2007. Conversion of methionine to cysteine in *Bacillus subtilis* and its regulation. *J. Bacteriol.* **189**:187–197.
  38. Jönsson, T. J., H. R. Ellis, and L. B. Poole. 2007. Cysteine reactivity and thiol-disulfide interchange pathways in AhpF and AhpC of the bacterial alkyl hydroperoxide reductase system. *Biochemistry* **46**:5709–5721.
  39. Kim, S. O., K. Merchant, R. Nudelman, W. F. Beyer, Jr., T. Keng, J. DeAngelo, A. Hausladen, and J. S. Stamler. 2002. OxyR: a molecular code for redox-related signaling. *Cell* **109**:383–396.
  40. Kosower, N. S., and E. M. Kosower. 1995. Diamide: an oxidant probe for thiols. *Methods Enzymol.* **251**:123–133.
  41. Lee, J. W., and J. D. Helmann. 2006. The PerR transcription factor senses H<sub>2</sub>O<sub>2</sub> by metal-catalysed histidine oxidation. *Nature* **440**:363–367.
  42. Lee, J. W., S. Soonsanga, and J. D. Helmann. 2007. A complex thiolate switch regulates the *Bacillus subtilis* organic peroxide sensor OhrR. *Proc. Natl. Acad. Sci. USA* **104**:8743–8748.
  43. Leelakriangsak, M., N. T. Huyen, S. Töwe, N. van Duy, D. Becher, M. Hecker, H. Antelmann, and P. Zuber. 2008. Regulation of quinone detoxification by the thiol stress sensing DUF24/MarR-like repressor, YodB in *Bacillus subtilis*. *Mol. Microbiol.* **67**:1108–1124.
  44. Leichert, L. L., and U. Jakob. 2006. Global methods to monitor the thiol-disulfide state of proteins in vivo. *Antioxid. Redox Signal.* **8**:763–772.
  45. Leichert, L. L. O., C. Scharf, and M. Hecker. 2003. Global characterization of disulfide stress in *Bacillus subtilis*. *J. Bacteriol.* **185**:1967–1975.
  46. Liebeke, M., D. C. Pöther, N. van Duy, D. Albrecht, D. Becher, F. Hochgräfe, M. Lalk, M. Hecker, and H. Antelmann. 2008. Depletion of thiol-containing proteins in response to quinones in *Bacillus subtilis*. *Mol. Microbiol.* **69**:1513–1529.
  47. Lillig, C. H., A. Potamitou, J. D. Schwenn, A. Vlamis-Gardikas, and A. Holmgren. 2003. Redox regulation of 3'-phosphoadenylylsulfate reductase from *Escherichia coli* by glutathione and glutaredoxins. *J. Biol. Chem.* **278**:22325–22330.
  48. Lithgow, J. K., E. J. Hayhurst, G. Cohen, Y. Aharonowitz, and S. J. Foster. 2004. Role of a cysteine synthase in *Staphylococcus aureus*. *J. Bacteriol.* **186**:1579–1590.
  49. Newton, G. L., K. Arnold, M. S. Price, C. Sherrill, S. B. delCardayre, Y. Aharonowitz, G. Cohen, J. Davies, R. C. Fahey, and C. Davis. 1996. Distribution of thiols in microorganisms: mycothiol is a major thiol in most actinomycetes. *J. Bacteriol.* **178**:1990–1995.
  50. Newton, G. L., N. Buchmeier, and R. C. Fahey. 2008. Biosynthesis and functions of mycothiol, the unique protective thiol of *Actinobacteria*. *Microbiol. Mol. Biol. Rev.* **72**:471–494.
  51. Newton, G. L., and R. C. Fahey. 1995. Determination of biothiols by bromobimane labeling and high-performance liquid chromatography. *Methods Enzymol.* **251**:148–166.
  52. Newton, G. L., M. Rawat, J. J. La Clair, V. K. Jothivasan, T. Budiarto, C. J. Hamilton, A. Claiborne, J. D. Helmann, and R. C. Fahey. 2009. Bacillithiol is an antioxidant thiol produced in bacilli. *Nat. Chem. Biol.* **5**:625–627.
  53. Nguyen, T. T., W. Eiamphungporn, U. Mader, M. Liebeke, M. Lalk, M. Hecker, J. D. Helmann, and H. Antelmann. 2009. Genome-wide responses to carbonyl electrophiles in *Bacillus subtilis*: control of the thiol-dependent formaldehyde dehydrogenase AdhA and cysteine proteinase YraA by the MerR-family regulator YraB (AdhR). *Mol. Microbiol.* **71**:876–894.
  54. Paget, M. S., and M. J. Buttner. 2003. Thiol-based regulatory switches. *Annu. Rev. Genet.* **37**:91–121.
  55. Pamp, S. J., D. Frees, S. Engelmann, M. Hecker, and H. Ingmer. 2006. Spx is a global effector impacting stress tolerance and biofilm formation in *Staphylococcus aureus*. *J. Bacteriol.* **188**:4861–4870.
  56. Ponce-de-Leon, M. M., and L. I. Pizer. 1972. Serine biosynthesis and its regulation in *Bacillus subtilis*. *J. Bacteriol.* **110**:895–904.
  57. Sommer, A., and R. R. Traut. 1974. Diagonal polyacrylamide-dodecyl sulfate gel electrophoresis for the identification of ribosomal proteins crosslinked with methyl-4-mercaptobutyrimidate. *Proc. Natl. Acad. Sci. USA* **71**:3946–3950.
  58. Soonsanga, S., J. W. Lee, and J. D. Helmann. 2008. Oxidant-dependent switching between reversible and sacrificial oxidation pathways for *Bacillus subtilis* OhrR. *Mol. Microbiol.* **68**:978–986.
  59. Soutourina, O., O. Poupel, J. Y. Coppée, A. Danchin, T. Msadek, and I. Martin-Verstraete. 2009. CymR, the master regulator of cysteine metabolism in *Staphylococcus aureus*, controls host sulphur source utilization and plays a role in biofilm formation. *Mol. Microbiol.* **73**:194–211.
  60. Stülke, J., R. Hanschke, and M. Hecker. 1993. Temporal activation of beta-glucanase synthesis in *Bacillus subtilis* is mediated by the GTP pool. *J. Gen. Microbiol.* **139**:2041–2045.
  61. Tanous, C., O. Soutourina, B. Raynal, M. F. Hullo, P. Mervelet, A. M. Gilles, P. Noiro, A. Danchin, P. England, and I. Martin-Verstraete. 2008. The CymR regulator in complex with the enzyme CysK controls cysteine metabolism in *Bacillus subtilis*. *J. Biol. Chem.* **283**:35551–35560.
  62. Tojo, S., T. Satomura, K. Kumamoto, K. Hirooka, and Y. Fujita. 2008. Molecular mechanisms underlying the positive stringent response of the *Bacillus subtilis* *ilv-leu* operon, involved in the biosynthesis of branched-chain amino acids. *J. Bacteriol.* **190**:6134–6147.
  63. Tsay, J. T., W. Oh, T. J. Larson, S. Jackowski, and C. O. Rock. 1992. Isolation and characterization of the beta-ketoacyl-acyl carrier protein synthase III gene (*fabH*) from *Escherichia coli* K-12. *J. Biol. Chem.* **267**:6807–6814.
  64. Wolf, C., F. Hochgräfe, H. Kusch, D. Albrecht, M. Hecker, and S. Engelmann. 2008. Proteomic analysis of antioxidant strategies of *Staphylococcus aureus*: diverse responses to different oxidants. *Proteomics* **8**:3139–3153.
  65. Zheng, M., X. Wang, B. Doan, K. A. Lewis, T. D. Schneider, and G. Storz. 2001. Computation-directed identification of OxyR DNA binding sites in *Escherichia coli*. *J. Bacteriol.* **183**:4571–4579.
  66. Zuber, P. 2004. SpX-RNA polymerase interaction and global transcriptional control during oxidative stress. *J. Bacteriol.* **186**:1911–1918.