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Proteomic Analysis of the Oxidative Stress Response Induced by Low-Dose Hydrogen Peroxide in *Bacillus anthracis*

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Anthrax is a bacterial disease caused by the aerobic spore-forming bacterium *Bacillus anthracis*, which is an important pathogen owing to its ability to be used as a terror agent. *B. anthracis* spores can escape phagocytosis and initiate the germination process even in antimicrobial conditions, such as oxidative stress. To analyze the oxidative stress response in *B. anthracis* and thereby learn how to prevent antimicrobial resistance, we performed protein expression profiling of *B. anthracis* strain HY1 treated with 0.3 mM hydrogen peroxide using a comparative proteomics-based approach. The results showed a total of 60 differentially expressed proteins; among them, 17 showed differences in expression over time. We observed time-dependent changes in the production of metabolic and repair/protection signaling proteins. These results will be useful for uncovering the metabolic pathways and protection mechanisms of the oxidative response in *B. anthracis*.

Key words: *Bacillus anthracis*, hydrogen peroxide, oxidative stress, proteomics

Anthrax is a bacterial disease caused by the aerobic spore-forming bacterium *Bacillus anthracis* (*B. anthracis*), which is an important pathogen in the *Bacillus cereus* group. *B. anthracis* can infect multiple hosts, including humans, and anthrax can be contracted *via* intradermal inoculation, ingestion, or inhalation of spores. During host infection, the spores enter macrophages by phagocytosis, but *B. anthracis* spores effectively germinate inside macrophages. When environmental conditions are severe, *B. anthracis* produces dormant spores that are more resistant to and better able to survive continuous exposure to diverse stresses. During phagocytosis, macrophages attempt to kill the invading bacteria with an antimicrobial oxidative burst, but *B. anthracis* initiates the spore germination process in response to components of an oxidative burst, such as superoxide ions [2]. Since 2001, anthrax has received much attention following anthrax attacks in the United States [18]. For *B. anthracis* spore decontamination, heat, desiccation, radiation, pressure, and oxidative chemicals such as hydrogen peroxide (H_2O_2) have been evaluated [19]. Many studies have aimed toward the prevention of antimicrobial resistance by focusing on the oxidative stress response in *B. anthracis* that allows cells to escape phagocytes.

Oxidative stress is generated by reactive oxygen species (ROS) such as superoxide anions, H_2O_2, and hydroxyl radicals (OH·) that result in damage to nucleic acids, cell membranes, and proteins [24]. Because of this damage, oxidative stress can inhibit cell growth and lead to cell death [20]. Against such endogenous and exogenous oxidative stress, pathogens have developed specific strategies to neutralize ROS [15]. They possess many antioxidant enzymes and repair activities, which are expressed at a basal level during growth, to defend against oxidative stress damage [24].

B. anthracis spores can survive and germinate in oxidative environments [24]. As an aerobe, *B. anthracis* cells cannot avoid the oxidative stress created by endogenous respiration. In *B. anthracis*, ROS are generated as by-products of endogenous respiration, and phagocytes subject the bacteria to an oxidative environment during infection [5]. YhgC, a protein involved in the staphylococcal stress response, protects *B. anthracis* from oxidative stress by up-regulating ClpP-2 and camelysin [11]. Tu *et al*. [27] compared the oxidative stress responses of *B. anthracis* and *B. subtilis* and found that KatB is the main vegetative catalase in *B. anthracis* and is regulated by the PerR repressor.

At present, proteomic techniques using two-dimensional gel electrophoresis (2DE) and mass spectrometry (MS) have been widely used to observe global differences in

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protein expression level in different environments [25]. Many advanced studies also used proteomic approaches to learn about the responses of several pathogens to oxidative stress [8, 14, 15, 25]. In one study, the superoxide and peroxide stress stimulons of *B. anthracis* strain UM23C1-2 were analyzed using a combined transcriptomics and proteomics approach. It was shown that the response to peroxide predominantly involves the induction of protection and repair mechanisms, including Dps-like proteins, Dps1 and Dps2, BCP proteins, ferredoxin oxidoreductase, and oxidoreductase [15]. Tu *et al*. [26] confirmed that a *dps2* mutant of *B. anthracis* was highly sensitive to H_2O_2 and that Dps2 protects cells from peroxide stress by inhibiting the iron-catalyzed production of hydroxyl radicals.

Nearly all reported studies of the oxidative stress response have used high concentrations of H_2O_2 and long treatment times. To analyze the oxidative stress response in *B. anthracis* that allows cells to escape phagocytes and resist antimicrobial environments, we used a comparative proteomics-based approach to examine the protein expression profile of *B. anthracis* strain HY1 isolated in Korea, treated with a relatively low concentration of H_2O_2. For safety experiment, we cured the pXO2 plasmid of the HY1 strain and used it in this study.

Materials and Methods

Strains, Culture Conditions, and Sample Preparation for 2DE

B. anthracis strain HY1 ($pXO1^+$, $pXO2^+$) was cultured aerobically in Luria–Bertani (LB) medium at 37°C in a shaking incubator. For the induction of oxidative stress, *B. anthracis* HY1 ($pXO1^+$, $pXO2^-$) was cultured until the OD540 nm reached 0.3, and H_2O_2 was added to these early-exponential-phase cells at a final concentration of 0.3 mM. For the preparation of cellular proteins, cell pellets were collected 1 and 2 h after H_2O_2 addition. The cells were treated with lysis buffer (9.5 M urea, 2.5% 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS), 40 mM dithiothreitol (DTT), 0.12% carrier ampholytes, 0.0012% bromophenol blue) and solubilized by sonication on ice.

2DE Gel Analysis and Spot Quantitation

To analyze cellular proteins, proteomic techniques were performed as previously described [9]. Isoelectric focusing (IEF) was performed using immobilized pH gradient (IPG) dry strips (4-10 NL IPG, 24 cm; Genomine, Korea) that were rehydrated for 16 h with 1.2 mg of sample at 20°C. After equilibration of the IPG strips, gel electrophoresis was conducted on sodium dodecyl sulfate (SDS) polyacrylamide gels (20 × 24 cm, 10–16%), and the gels were stained with Coomassie brilliant blue [22, 23].

Quantitative analysis of digitized images was performed using PDQuest (version 7.0; BioRad Laboratories, USA) software, and protein spots were selected based on significant expression variation, defined as greater than 2-fold differences between the control and H_2O_2-treated samples [21].

In-Gel Digestion, MALDI-TOF MS Analysis, and Protein Database Search

Protein spots showing expression differences under oxidative stress were selected for further characterization using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The selected protein spots were digested enzymatically in the gel as previously described by Seo *et al*. [21] using porcine trypsin (Promega, USA). The gel pieces were washed three times with acetonitrile and ammonium bicarbonate to remove the solvents. They were then rehydrated with trypsin (8–10 ng/μl) and incubated for 16 h at 37°C. To terminate the proteolytic reaction, 5 μl of 0.5% trifluoroacetic acid was added. The peptides were extracted with 50% aqueous acetonitrile.

Peptide analysis was performed using an Ultraflex MALDI-TOF mass spectrometer (Bruker Daltonics, Germany). After the peptides were loaded on the plate, they were evaporated with an N_2 laser at 337 nm using a delayed extraction approach and were accelerated with a 20 kV injection pulse for time-of-flight analysis. From the peptide fragment sizes, we predicted protein identities using a search program, Profound, developed by Rockefeller University (http://prowl.rockefeller.edu/prowl-cgi/profound.exe). We searched against the freely accessible NCBI online database (results were based on a Z score of greater than 1.65). The search parameters were a maximum of one missed cleavage by trypsin, fixed modification of oxidation, charge state of +1, and a mass tolerance of ±1 Da.

Real-Time Reverse-Transcription-PCR (RT-PCR) Analysis

Bacterial RNA was extracted from *B. anthracis* cultures treated with H_2O_2 for 1 or 2 h using the RNAprotect Bacterial Reagent (Qiagen, Germany) and the RNeasy Mini Kit (Qiagen, Germany). Bacterial RNA (4 μg) was reverse transcribed into cDNA using SuperScript II reverse transcriptase (Invitrogen, USA) and random primers. Real-time RT-PCR was performed as described by Kim *et al*. [10]. To perform real-time PCR, 10 μl of SYBR Premix Ex Taq II (Takara BIO, Japan), 0.4 μl of ROX, 5 pmol of forward primer, 5 pmol of reverse primer, and 0.4 μl of cDNA were mixed with water to a final volume of 20 μl. The mixture was amplified using a 7500 Fast

Table 1. Primers used in the real-time RT-PCR.

Gene	Protein	Primer sequences	Product size (bp)
BA_1330	Acetoacetyl CoA reductase	5'-GGAACGCGTAATTGACGTGA-3' 5'-CCGCGCTTGTCGTATTAAACA-3'	53
BA_4383	2-Oxoisovalerate dehydrogenase subunit beta	5'-CATCATGCCAGCAGTAAACCA-3' 5'-CAAATGGCGCACGAACTGTA-3'	101
BA_4385	Dihydrolipoamide dehydrogenase	5'-TCTCTCCGATGCCAGGGAC-3' 5'-CGGACGAGAACCTGTTGCA-3'	101

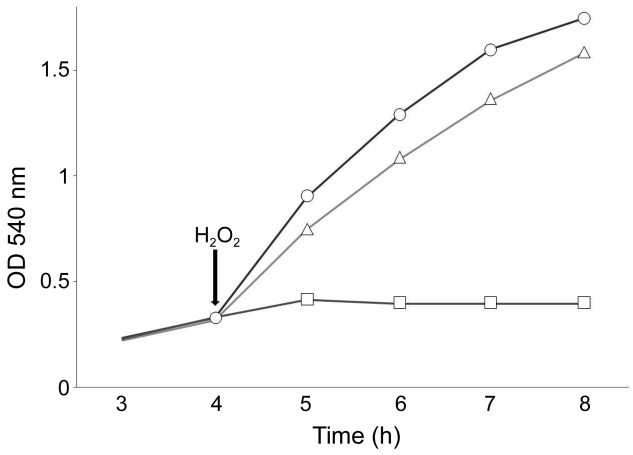

Fig. 1. Growth curve of *B. anthracis* strain HY1 in the presence of H_2O_2 to make an optimal concentration of H_2O_2 as low-dose oxidative stress.
Cells were grown in LB medium at 37°C. When the culture reached an OD_{540} of 0.3, different concentrations (0.1~1.0 mM) of H_2O_2 were added (data were not shown). For further analysis, a final concentration of 0.3 mM H_2O_2 was selected as the optimal condition. All experiments were repeated three times (untreated control, ○; 0.3 mM, △; 1.0 mM, □; $*p < 0.005$).

Real-Time PCR system (Applied Biosystems, USA) with an initial denaturation at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min [7]. The cycle number at which a statistically significant increase in the expression of each gene was first detected (threshold cycle, C_t) was then normalized to the C_t for 16S rRNA, a housekeeping gene. Gene expression patterns were calculated for the growth phase at 37°C or at different temperatures using the $2^{-\Delta\Delta CT}$ method. The primer design was performed using the Primer Express program (Applied Biosystems, USA); the primer sequences are listed in Table 1.

RESULTS

Growth Curve of *B. anthracis* in the Presence of Hydrogen Peroxide

When the culture reached an OD_{540} of 0.3, different concentrations (0.1~1.0 mM) of H_2O_2 were added (data not shown). From several growth experiment results, we selected a final concentration of 0.3 mM H_2O_2. We selected this concentration because it caused slight inhibition of cell growth but did not kill the cells. By testing H_2O_2 concentrations between 0.1 and 1.0 mM, approximately 10% growth inhibition was observed at 0.3 mM, leading to its selection as the final concentration (Fig. 1).

Proteomic Analysis

Cellular proteins of *B. anthracis* HY1, which is a strain isolated in Korea, were extracted from an untreated control culture and from cultures treated for 1 or 2 h with H_2O_2 and were analyzed using proteomic techniques. A total protein mass of 1.2 mg was analyzed by 2DE analysis, and approximately 800 spots, on average, were observed per Coomassie brilliant blue-stained gel (Fig. 2). Image analyses were performed to detect increased or decreased

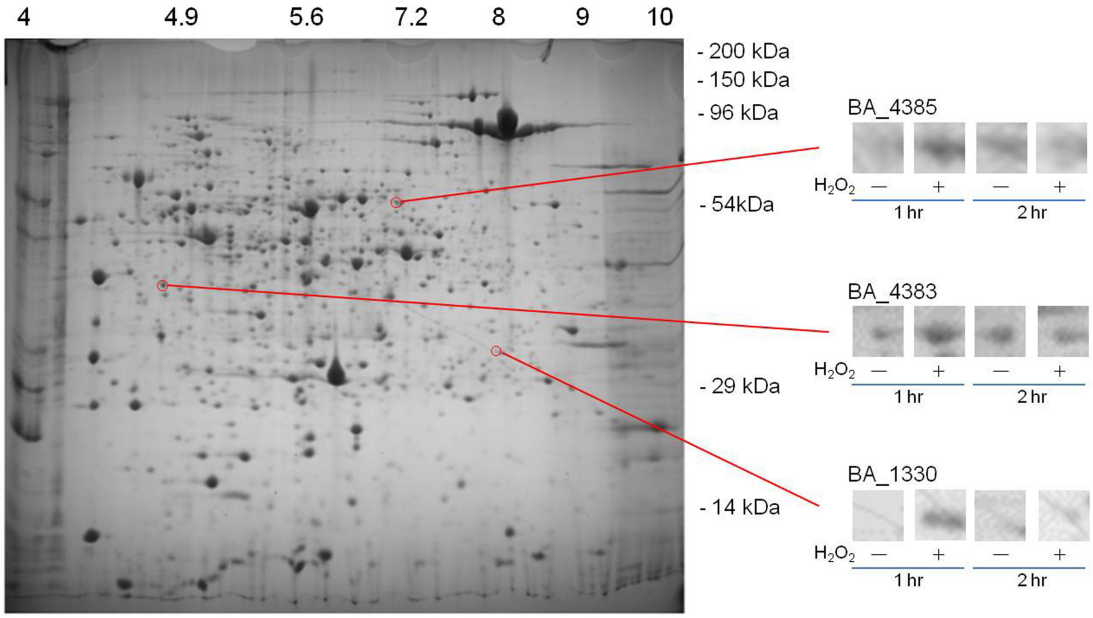

Fig. 2. Two-dimensional gel electrophoresis of *B. anthracis* HY1 cellular proteins.
The three proteins indicated by circles on the gel show different intensities between 1 and 2 h of treatment: spot 1, BA_4385; spot 2, BA_4383; spot 3, BA_1330.

spot intensity in the 1 h H_2O_2-treated sample compared with the control.

With 1 h of treatment, a total of 28 proteins were affected by H_2O_2; among them, 23 proteins were upregulated and 5 were downregulated. With 2 h of treatment, a total of 52 proteins were affected by H_2O_2; among them, 13 proteins were upregulated and 39 proteins were downregulated. Together, a total of 60 unique proteins were affected by H_2O_2 in at least one treatment period. Among them, 43 proteins showed similar patterns regardless of treatment time, with 12 upregulated proteins and 31 downregulated proteins. However, 17 proteins were expressed differently between the treatment periods. Among them, 16 proteins were upregulated after 1 h of treatment but were dramatically downregulated after 2 h. The remaining protein showed the opposite trend. From these data, we attempted to observe changes in metabolic and repair/protection signaling over time. The information regarding these proteins and their cellular functions are listed in Tables 2, 3, and 4.

Real-Time RT-PCR Analysis

Oxidoreductase is an important component of redox reaction and three differentially regulated genes (BA_1330, BA_4383, and BA_4385) that have the function of oxidoreductase were selected from the proteomic data for validation by real-time RT-PCR (Fig. 3). Each H_2O_2-treated sample was

Table 2. Differentially expressed proteins after 1 h of treatment with 0.3 mM H_2O_2.

Accession no.	Protein	Cellular function	Fold change (1 h)
BA_0107	Elongation factor G	GTP binding; GTPase activity translation; Elongation factor activity	739.1
BA_0309	1-Pyrroline-5-carboxylate dehydrogenase	1-Pyrroline-5-carboxylate dehydrogenase activity; Oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	2.2
BA_0345	Alkyl hydroperoxide reductase subunit C	Peroxidase activity; Peroxiredoxin activity	17.9
BA_0467	Prophage LambdaBa04, prohead protease	Peptidase activity	801.3
BA_0468	Prophage LambdaBa04, major capsid protein	-	1,134.1
BA_0469	Hypothetical protein	-	3,362
BA_0481	Phage minor structural protein	-	1,161
BA_0482	Hypothetical protein	-	1,433.4
BA_0485	Prophage LambdaBa04, glycosyl hydrolase 25	Cation binding; Lysozyme activity	3,055.8
BA_1154	Ornithine-oxo-acid transaminase	Ornithine-oxo-acid transaminase activity; Pyridoxal phosphate binding	1.5
BA_2107	Formate-tetrahydrofolate ligase	ATP binding formate-tetrahydrofolate; Ligase activity	2.3
BA_2267	Alcohol dehydrogenase	Alcohol dehydrogenase (NAD) activity; Nucleotide binding; Zinc ion binding	2.8
BA_2356	Hydroxyisobutyryl-CoA hydrolase	Isomerase activity	2
BA_3338	S-Layer protein	-	2.2
BA_3609	Aldehyde dehydrogenase	Aldehyde dehydrogenase (NAD) activity	1.7
BA_3915	Recombinase A	ATP binding; DNA-dependent ATPase activity; Single-stranded DNA binding	6.6
BA_3935	Dihydrodipicolinate synthase	Dihydrodipicolinate synthase activity	2.7
BA_4308	Purine nucleoside phosphorylase	Purine-nucleoside phosphorylase activity	-1.7
BA_4875	Universal stress protein family	-	15.8
BA_4890	Thiol peroxidase	Thioredoxin peroxidase activity	-1.9
BA_4893	Inorganic polyphosphate/ATP-NAD kinase	ATP binding; NAD+ kinase activity	2
BA_5125	L-Lactate dehydrogenase	L-Lactate dehydrogenase activity; Nucleotide binding	5.3
BA_5365	Phosphoglyceromutase	Manganese ion binding; Phosphoglycerate mutase activity	-5.5
BA_5366	Triose-phosphate isomerase	Triose-phosphate isomerase activity	-1.7
BA_5563	PTS system glucose-specific transporter subunit IIA	Kinase activity; Sugar:hydrogen symporter activity	-2

Cellular functions were found with UniProtKB (http://www.uniprot.org/uniprot/).

Table 3. Differentially expressed proteins after 2 h of treatment with 0.3 mM H_2O_2.

Accession no.	Protein	Cellular function	Fold change (2 h)
BA_0010	Pyridoxal biosynthesis lyase PdxS	Lyase activity	-2.7
BA_0107	Elongation factor G	GTP binding; GTPase activity translation; Elongation factor activity	3.4
BA_0196	Aldo/keto reductase family oxidoreductase	Oxidoreductase activity	-4
BA_0309	1-Pyrroline-5-carboxylate dehydrogenase	1-Pyrroline-5-carboxylate dehydrogenase activity; Oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	-2,000
BA_0399	Tellurium resistance protein	-	-1.9
BA_0459	Hypothetical protein	-	5
BA_0467	Prophage LambdaBa04, prohead protease	Peptidase activity	5.7
BA_0468	Prophage LambdaBa04, major capsid protein	-	2.4
BA_0469	Hypothetical protein	-	9.6
BA_0481	Phage minor structural protein	-	6
BA_0482	Hypothetical protein	-	-1,400
BA_0485	Prophage LambdaBa04, glycosyl hydrolase 25	Cation binding; Lysozyme activity	3.1
BA_0492	Phage minor structural protein	Amino acid transmembrane transporter activity	1,033.8
BA_0509	Formate acetyltransferase	Formate C-acetyltransferase activity	-28
BA_0620	2-Amino-3-ketobutyrate coenzyme A ligase	Pyridoxal phosphate binding; Transferase activity	-2.1
BA_0670	Transaldolase	Sedoheptulose-7-phosphate:D-glyceraldehyde-3-phosphate glyceronetransferase activity	-3
BA_1132	Isocitrate lyase	Isocitrate lyase activity	-2
BA_1154	Ornithine-oxo-acid transaminase	Ornithine-oxo-acid transaminase activity; Pyridoxal phosphate binding	-2.5
BA_1270	2-Oxoglutarate dehydrogenase E1 component	Oxoglutarate dehydrogenase activity; Thiamine pyrophosphate binding	2.5
BA_1327	phaP protein	-	-1.7
BA_1330	Acetoacetyl-CoA reductase	Acetoacetyl-CoA reductase activity; Nucleotide binding	-3.5
BA_1583	Hypothetical protein	-	-2.4
BA_2107	Formate-tetrahydrofolate ligase	ATP binding formate-tetrahydrofolate; Ligase activity	-3.3
BA_2267	Alcohol dehydrogenase	Alcohol dehydrogenase (NAD) activity	-14
BA_2276	Azoreductase	-	-2.5
BA_2308	spo0M gene product (predicted)	-	-12
BA_2356	Hydroxyisobutyryl-CoA hydrolase	Isomerase activity	-1.7
BA_3609	Aldehyde dehydrogenase	Aldehyde dehydrogenase (NAD) activity	-700
BA_3660	Serine protease	Serine-type endopeptidase activity; Serine-type peptidase activity	-1.9
BA_3909	2-Oxoglutarate ferredoxin oxidoreductase subunit beta	Oxidoreductase activity; Thiamine pyrophosphate binding	-2.2
BA_3915	Recombinase A	ATP binding; DNA-dependent ATPase activity; Single-stranded DNA binding	3.9
BA_3935	Dihydrodipicolinate synthase	Dihydrodipicolinate synthase activity	-1.7
BA_4181	Dihydrolipoamide dehydrogenase	Dihydrolipoyl dehydrogenase activity; Flavin adenine dinucleotide binding	2.2
BA_4226	Glycine cleavage system aminomethyltransferase T	DNA binding; Sequence-specific DNA binding transcription factor activity	-3
BA_4308	Purine nucleoside phosphorylase	Purine-nucleoside phosphorylase activity	-5
BA_4315	Acetyltransferase	*N*-Acetyltransferase activity	-2.5
BA_4383	2-Oxoisovalerate dehydrogenase subunit beta	3-Methyl-2-oxobutanoate dehydrogenase activity	-1.8
BA_4385	Dihydrolipoamide dehydrogenase	Dihydrolipoyl dehydrogenase activity; Flavin adenine dinucleotide binding	-2

Table 3. Continued.

Accession no.	Protein	Cellular function	Fold change (2 h)
BA_4759	Electron transfer flavoprotein subunit alpha	Electron carrier activity; Flavin adenine dinucleotide binding	-3.5
BA_4761	Enoyl-CoA hydratase	isomerase activity	-9
BA_4875	Universal stress protein family	-	-5.5
BA_4893	Inorganic polyphosphate/ATP-NAD kinase	ATP binding; NAD+ kinase activity	-2.7
BA_5109	Naphthoate synthase	1,4-Dihydroxy-2-naphthoyl-CoA synthase activity	-1.75
BA_5153	Phosphoglucomutase/phosphomannomutase	Intramolecular transferase activity; Phosphotransferases; Magnesium ion binding	-2.8
BA_5213	SufB-like Fe–S cluster assembly protein	-	2.4
BA_5314	Trysosyl-tRNA synthetase	ATP binding; RNA binding; Tyrosine-tRNA ligase activity	-2
BA_5369	Glyceraldehyde-3-phosphate dehydrogenase	NAD binding; NADP binding; Oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	-2
BA_5563	PTS system glucose-specific transporter subunit IIA	Kinase activity; Sugar: hydrogen symporter activity	-1.9
BA_5576	Fructose 1,6-bisphosphatase II	Hydrolase activity; Metal ion binding	-4.3
BA_5580	Fructose-bisphosphate aldolase	Fructose-bisphosphate aldolase activity; Zinc ion binding	-5

Cellular functions were found with UniProtKB (http://www.uniprot.org/uniprot/).

compared with the control group, and the mRNA level was analyzed. A comparison of the gene expression between 1 and 2 h revealed that the mRNA level of these genes decreased over time. The *B. anthracis* culture treated with

Table 4. Differentially expressed proteins between 1 and 2 h of treatment with H_2O_2.

Gene	Protein	Cellular function	Fold change	
			1 h	2 h
BA_0482	Hypothetical protein BA_0482	-	1,433	-1,400
BA_2267	Alcohol dehydrogenase	Alcohol dehydrogenase (NAD) activity; Nucleotide binding; Zinc	2.8	-14
BA_2356	Hydroxyisobutyryl-CoA hydrolase	Isomerase activity	2.0	-1.7
BA_1154	Ornithine-oxo-acid transaminase	Ornithine-oxo-acid transaminase activity; Pyridoxal phosphate binding	1.5	-2.5
BA_3935	Dihydrodipicolinate synthase	Dihydrodipicolinate synthase activity	2.7	-1.7
BA_3338	S-Layer protein	-	**2.2**	-1.5
BA_2107	Formate-tetrahydrofolate ligase	ATP binding formate-tetrahydrofolate; Ligase activity	2.3	-3.3
BA_0309	1-Pyrroline-5-5carboxylate dehydrogenase	1-Pyrroline-5-carboxylate dehydrogenase activity; Oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	2.2	-2,000
BA_3609	Aldehyde dehydrogenase	Aldehyde dehydrogenase (NAD) activity	1.7	-700
BA_4875	Universal stress protein family	-	15.8	-5.5
BA_4893	Inorganic polyphosphate/ATP-NAD kinase	ATP binding; NAD+ kinase activity	2.0	-2.7
BA_0107	Elongation factor G	GTP binding; GTPase activity translation; Elongation factor activity	739.1	3.4
BA_0467	Prophage LambdaBa04, prohead protease	Peptidase activity	801.3	5.7
BA_0469	Hypothetical protein BA_0469	-	3362	9.6
BA_0481	Phage minor structural protein	-	1,161.0	6.0
BA_0485	Prophage LambdaBa04, glycosyl hydrolase 25	Cation binding; Lysozyme activity	3,055.8	3.1
BA_4181	Dihydrolipoamide dehydrogenase	Dihydrolipoyl dehydrogenase activity; Flavin adenine dinucleotide binding	-	2.2

Cellular functions were found with UniProtKB (http://www.uniprot.org/uniprot/).

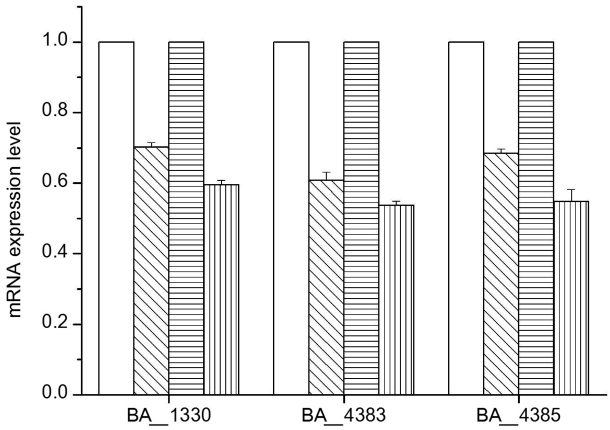

Fig. 3. Expression patterns of mRNAs encoding oxidoreductase activity are affected by H_2O_2 in *B. anthracis*.
Bacterial RNA extraction from *B. anthracis* was performed after 1 or 2 h of H_2O_2 treatment, and the resulting mRNA was analyzed by real-time RT-PCR (Control 1 h, □; H_2O_2 1 h, ▨; Control 2 h, ▤; H_2O_2 2 h, ▥). The mRNA expression level of *B. anthracis* treated with H_2O_2 decreased over time. All data were repeated three times (*BA_1330 and BA_4383, $p < 0.002$; BA_4385, $p < 0.009$).

H_2O_2 for 2 h showed a decrease of nearly 50% compared with the control, which is a further decrease from the approximately 20% decrease after 1 h of treatment.

DISCUSSION

Oxidative stress has an important role in killing invading bacteria during phagocytosis, but *B. anthracis* can escape the phagocytes and initiate the spore germination process under oxidative stress conditions [2]. Oxidative stress-inducing compounds, such as H_2O_2, have been evaluated as *B. anthracis* spore decontaminants [19]. To analyze the oxidative stress response in *B. anthracis* that allows the bacterial cells to escape phagocytes and resist antimicrobial conditions, we chose to use 0.3 mM H_2O_2, which caused approximately 10% growth inhibition. We analyzed the protein expression profile of *B. anthracis* strain HY1 treated with 0.3 mM H_2O_2 using a comparative proteomics-based approach.

Many proteins related to protection and repair were induced in 1 h and/or reduced in 2 h by H_2O_2, including alkyl hydroperoxide reductase subunit C (AhpC, BA_0345), azoreductase (BA_2276), 2-oxoglutarate ferredoxin oxidoreductase subunit beta (BA_3909), tellurium resistance protein (BA_0399), and recombinase A (RecA, BA_3915). Those proteins have been observed in previous studies and are related to protection and repair [4, 12, 15]. In the case of AhpC, it has been observed that it could be more damaged with acidic forms in *Staphylococcus aureus*. Homologs are present in other species as part of a thiol-specific antioxidant

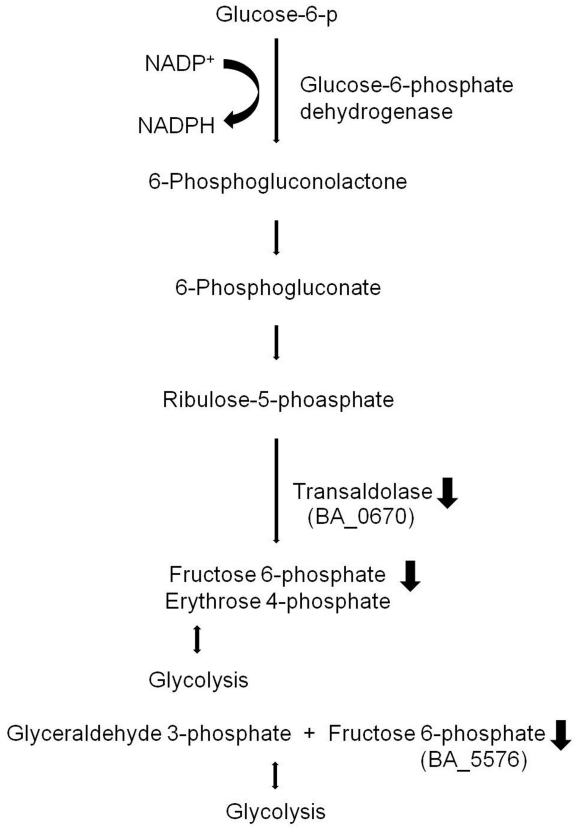

Fig. 4. The pentose phosphate pathway is able to increase resistance against an increasingly oxidative environment in the cytosol caused by oxidative stress.
A total of 5 proteins related to this pathway were observed in the proteomic data (BA_0670, BA_3609, BA_5153, BA_5576, and BA_5580). These proteins were downregulated after 2 h and are displayed with thick narrows.

protein family related to cysteine-containing hydrophobic motifs that have a catalytic antioxidative activity [1]. The expression of AhpC was upregulated by 1 h of oxidative stress, which is a finding that is consistent with the studies of Pohl *et al*. [15]. These findings show that proteins related to protection and repair are upregulated to protect from H_2O_2 stress during the first hour of treatment, but some of these proteins are downregulated after the initial period of defense to return the cells to their original condition.

Dihydrodipicolinate synthase (DapA2, BA_3935) and a universal stress protein family member (BA_4875) were upregulated in *B. anthracis* by oxidative stress after 1 h, but downregulated after 2 h. The structure and function of DapA2 are already known [6, 17, 28]. DapA2 catalyzes the first committed step of the biosynthetic pathway that produces the essential amino acids meso-diaminopimelate (DAP) and lysine [6]. In *Bacillus subtilis*, dipicolinate synthase produces dipicolinate for protection in a superoxide dismutase (SOD) mutant strain [13]. Therefore, in *B. anthracis*, DapA2 may produce dihydrodipicolinate to

protect the cells for the first hour of H_2O_2 treatment, but it is then downregulated after the initial period of defense to return the cells to their original condition. Another example is the universal stress protein family member (BA_4875); it has a role as a response regulator under oxygen starvation conditions in *Mycobacterium smegmatis* [14].

Many proteins related to metabolism were also expressed when cells were exposed to H_2O_2. H_2O_2 induces radical changes in the expression of genes that encode metabolic enzymes. Among the most important of these is the pentose phosphate pathway, which is able to increase resistance against an increasingly oxidative environment in the cytosol (Fig. 4). Enzymes in this pathway include transaldolase (BA_0670), which links the pentose phosphate pathway to glycolysis; aldehyde dehydrogenase (BA_3609), which is a polymorphic enzyme responsible for the oxidation of aldehydes to carboxylic acids; phosphoglucomutase/phosphomannomutase (BA_5153), which participates in fructose and mannose metabolism; fructose 1,6-bisphosphatase II (BA_5576), which catalyzes the reverse of the reaction that is catalyzed by phosphofructokinase in glycolysis; and fructose-bisphosphate aldolase (BA_5580), which catalyzes a reversible reaction that splits fructose 1,6-bisphosphate (an aldol) into the triose phosphates dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP). The genes related to the pentose phosphate pathway were downregulated after 2 h. These findings show that proteins related to oxidative stress in the cytosol were downregulated after an initial defense period to return the cells to their original condition.

Several oxidoreductase proteins exhibited notable differences. Three proteins, namely acetoacetyl-CoA reductase (PhbB, BA_1330), 2-oxoisovalerate dehydrogenase subunit beta (BfmbAb, BA_4383), and dihydrolipoamide dehydrogenase (BfmbC, BA_4385) were downregulated compared with the control. In other words, those proteins were inhibited in their catalytic function and do not make products such as acyl and acetyl-CoA, in contrast to the enzymes that were induced to reduce oxidative stress.

Among several oxidoreductase proteins, an aldo/keto reductase family oxidoreductase (BA_0196) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, gap2, BA_5369) were upregulated after 1 h, but dramatically changed to being downregulated after 2 h. The aldo-keto reductase family contains a number of related monomeric NADPH-dependent oxidoreductases [3], and GAPDH catalyzes the conversion of glyceraldehyde-3-phosphate and is inactivated by oxidant treatment [16]. Therefore, the metabolic pathway used oxidoreductases for the first hour of oxidative stress, but then shifted to the opposite direction after 2 h because the reaction was completed.

Our results showed that many proteins in *B. anthracis* were upregulated or downregulated when cells were exposed to 0.3 mM H_2O_2. We found that some proteins recovered from oxidative stress and that the expression level of such proteins also recovered. These finding may be important for understanding the resistance mechanisms of *B. anthracis*. In future studies, a more detailed comparison of the expression at multiple time points will contribute to the understanding of the survival mechanism of *B. anthracis* against oxidative stress.

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