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Comparative Transcriptomic and Phenotypic Analysis of the Responses of *Bacillus cereus* to Various Disinfectant Treatments▽†

Mara Ceragioli,[1,2,3] Maarten Mols,[1] Roy Moezelaar,[3,4] Emilia Ghelardi,[5] Sonia Senesi,[2] and Tjakko Abee[1,3]*

Laboratory of Food Microbiology, Wageningen University, Wageningen, Netherlands[1]; Dipartimento di Biologia, University of Pisa, Pisa, Italy[2]; Top Institute of Food and Nutrition, Wageningen, Netherlands[3]; Wageningen UR, Food and Biobased Research, Wageningen, Netherlands[4]; and Dipartimento di Patologia Sperimentale BMIE, University of Pisa, Pisa, Italy[5]

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Antimicrobial chemicals are widely applied to clean and disinfect food-contacting surfaces. However, the cellular response of bacteria to various disinfectants is unclear. In this study, the physiological and genome-wide transcriptional responses of *Bacillus cereus* ATCC 14579 exposed to four different disinfectants (benzalkonium chloride, sodium hypochlorite, hydrogen peroxide, and peracetic acid) were analyzed. For each disinfectant, concentrations leading to the attenuation of growth, growth arrest, and cell death were determined. The transcriptome analysis revealed that *B. cereus*, upon exposure to the selected concentrations of disinfectants, induced common and specific responses. Notably, the common response included genes involved in the general and oxidative stress responses. Exposure to benzalkonium chloride, a disinfectant known to induce membrane damage, specifically induced genes involved in fatty acid metabolism. Membrane damage induced by benzalkonium chloride was confirmed by fluorescence microscopy, and fatty acid analysis revealed modulation of the fatty acid composition of the cell membrane. Exposure to sodium hypochlorite induced genes involved in metabolism of sulfur and sulfur-containing amino acids, which correlated with the excessive oxidation of sulfhydryl groups observed in sodium hypochlorite-stressed cells. Exposures to hydrogen peroxide and peracetic acid induced highly similar responses, including the upregulation of genes involved in DNA damage repair and SOS response. Notably, hydrogen peroxide- and peracetic acid-treated cells exhibited high mutation rates correlating with the induced SOS response.

Bacillus cereus is a Gram-positive, rod-shaped bacterium, widespread in nature. The ability of *B. cereus* to produce endospores accounts for its ubiquitous occurrence in natural environments as well as for the high frequency of its isolation from various kinds of contaminated raw and processed food products (3, 29). *B. cereus* is the causative agent of two types of toxin-associated food-borne diseases: emetic and diarrheal syndromes (50). The emetic syndrome is an intoxication caused by the thermostable emetic toxin cereulide. The emetic toxin is produced by vegetative *B. cereus* cells in food before ingestion and remains active upon stomach transit. It is toxic to mitochondria by acting as a potassium ionophore (38) and has been reported to inhibit human natural killer cells (43). The diarrheal syndrome is caused by enterotoxins secreted by vegetative cells in the small intestine, where they can act by disrupting the integrity of the membrane of epithelial cells (56).

To prevent (re)contamination of food by bacteria that are involved in spoilage and/or food-borne diseases, food-contacting surfaces are cleaned regularly by the application of antimicrobial chemicals, such as benzalkonium chloride (BC), sodium hypochlorite (SH), hydrogen peroxide (HP), and peracetic acid (PAA) (9). BC is widely used as surface disinfectant, antiseptic, and preservative. It belongs to the quaternary ammonium compounds (QACs), which are membrane-active agents (36). Their antibacterial activity has been suggested to be associated with the loss of integrity of the cytoplasmic membrane and subsequent leakage of intracellular material upon adsorption by bacterial cells (49). SH is extensively used in the food processing industry (66). It dissolves in water, dissociating in Na^+ and the hypochlorite ion (OCl^-). In aqueous solutions, OCl^- establishes an equilibrium with hypochlorous acid (HOCl) (10). The mode of action of SH has not been clarified yet; however, it has been proposed that exposure to SH elicits responses similar to those elicited by HP treatments by generating superoxide anions and hydroxyl radicals ($OH^\bullet$) and that this reactive oxygen species largely accounts for the major bactericidal activity of SH (20). HP is commonly used as a disinfectant and is considered environmentally friendly because it breaks down into oxygen and water (9). It induces a burst of free $OH^\bullet$ radicals that can damage DNA and may ultimately result in cell death (15, 18). PAA is formed by the reaction of HP and acetic acid and is considered a more potent biocide than HP, because it shows antibacterial activity at lower concentrations than HP alone (9). Similar to HP, PAA also decomposes to safe waste products (acetic acid and oxygen). Additionally, it has the advantage of being resistant to inactivation by peroxidases and it remains active in the presence of organic matter (35). Furthermore, it can be used in wide temperature and pH ranges (31, 32).

The aim of this study was to analyze the general and specific

* Corresponding author. Mailing address: Laboratory of Food Microbiology, Wageningen University, Bomenweg 2, 6703 HD Wageningen, Netherlands. Phone: 31-317484981. Fax: 31-317484978. E-mail: Tjakko.Abee@wur.nl.

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transcriptome responses of *B. cereus* ATCC 14579 upon exposure to various disinfectant treatments, such as BC, SH, HP, and PAA, by using a genome-wide comparative transcriptional approach. In addition, phenotypic analyses of disinfectant-exposed cells were performed to support the results obtained from the transcriptome analyses.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and disinfectants. In this study, the sequenced *B. cereus* type strain ATCC 14579 obtained from the American Type Culture Collection (ATCC) and its *rsbY* (61) and *rsbK* (17) mutant derivatives, which are unable to activate σ^B, were used. *B. cereus* overnight cultures, grown in brain heart infusion (BHI) (Becton Dickinson, France) broth at 30°C, were used to inoculate 20 ml of BHI. The cultures were incubated at 30°C with shaking at 200 rpm for approximately 3 h until the optical density at 600 nm (OD_{600}) reached ~0.5 (~8 log CFU ml^{-1}; Novaspek II; Pharmacia Biotek, United Kingdom), which corresponded to the mid-exponential phase of growth. These cultures were subsequently treated with different concentrations of BC (0.5 to 7.0 $\mu g\ ml^{-1}$; Fluka, Sigma-Aldrich, St. Louis, MO), SH (50 to 750 $\mu g\ ml^{-1}$; Sigma-Aldrich, Germany), HP (0.05 to 0.2 mM; Merck, Germany), or PAA (0.5 to 250 $\mu g\ ml^{-1}$; Fluka) by adding highly concentrated (≤0.5% [vol/vol] of the final volume) stock solutions of each disinfectant prepared in sterile demineralized water immediately before use. An aliquot of each culture was collected before addition of the disinfectants (t_0) and after 10 (t_{10}), 30 (t_{30}), and 60 (t_{60}) min of exposure to the compounds. The physiological response of the bacterial cells was investigated by enumeration of viable cells. Serial dilutions of cultures were made in peptone physiological salt solution (PPS) (1 g neutralized bacteriological peptone [Oxoid, England] per liter and 8.5 g NaCl per liter) and plated on BHI agar plates (15 g agar [Oxoid] per liter). Colonies were counted after 24 h of incubation at 30°C. In order to isolate RNA, 100-ml cultures were used. Upon reaching the mid-exponential growth phase, 20 ml of the culture was collected and used to extract RNA for the unstressed sample (t_0). The remaining 80 ml was exposed to BC (1, 2, or 5 $\mu g\ ml^{-1}$), SH (300, 400, or 500 $\mu g\ ml^{-1}$), HP (0.05 or 0.2 mM), or PAA (10 or 100 $\mu g\ ml^{-1}$) and further incubated. After 10 (t_{10}) and 30 (t_{30}) min of exposure to the disinfectants, 20 ml of stressed cultures was collected. The pH (PHM240 pH/ION meter; Radiometer, Denmark) and OD_{600} (Novaspek II; Pharmacia Biotek) of the exposed cultures were periodically monitored.

RNA isolation and cDNA synthesis. RNA samples were obtained from unstressed and stressed cultures that were prepared as described above. Cultures were pelleted by centrifugation (11,000 rpm for 30 s at 4°C; Centrifuge 5804R, Eppendorf, Germany). The cell pellet was resuspended in 1 ml TRI-reagent (Ambion, United Kingdom) and immediately frozen in liquid nitrogen. Two biological replicates were performed under identical conditions. RNA isolation was performed as previously described (61), and RNA concentrations were measured by UV spectrophotometer readings at 260 and 280 nm (BioPhotometer, Eppendorf, Germany). Cyclic DNA (cDNA) synthesis and the Cy3 and Cy5 labeling of cDNAs were performed as previously described (62). After purification with the CyScribe GFX Purification kit (GE Healthcare, Belgium), labeled cDNAs were quantified by UV spectrophotometry.

Microarray hybridization and data analysis. Labeled cDNAs from the t_0 sample and the corresponding t_{10} or t_{30} samples were hybridized on custom-made Agilent *B. cereus* ATCC 14579 microarrays (GEO accession number GPL9493). Hybridization and subsequent washing of the slides were performed according to the manufacturer's instructions. The microarray slides were scanned using an Agilent microarray scanner (G2565BA), and data were processed with Agilent's Feature Extraction software (version 8.1.1.1). The normalized data for each spot from the microarrays were analyzed for statistical significance using the Web-based VAMPIRE microarray suite (24). *P* values were calculated for multiple comparisons, and spots with a false discovery rate ($P < 0.05$) were considered statistically significant. Expression ratios were calculated by integrating the data of spots representing the same open reading frame, and genes were considered differentially expressed when all spots were significantly differentially expressed between samples. The average expression ratios were calculated by averaging the values of the duplicate hybridizations, which were performed with the dyes swapped. An open reading frame was considered significantly induced or repressed when expression was ≥2.0-fold different from that of the reference sample. The analysis was performed as described by Mols et al. (40) using GeneMaths XT software (version 1.6.1; Applied Maths, Belgium). For gene annotation and metabolic routes, KEGG databases and the Simpheny software package (Genomatica, CA) were used.

Fluorescence microscopy. Samples for fluorescence microscopy were collected before (t_0) and 10 (t_{10}) and 30 (t_{30}) min after the addition of the various disinfectants. An aliquot of 1 ml culture of each condition analyzed was briefly spun down and resuspended in phosphate-buffered saline (PBS) (138 mM NaCl, 2.7 mM KCl, 140 mM Na_2HPO_4, 1.8 mM KH_2PO_4, adjusted to pH 7.4 with HCl). Cell suspensions were labeled for 15 min at 30°C with SYTO-9 (membrane-permeant, green fluorescent nucleic acid probe) and propidium iodide (PI) (red fluorescent nucleic acid probe) probes (Molecular Probes, Netherlands) at the final concentrations of 3.34 and 20 μM, respectively. Stained cells were analyzed using a Zeiss Axioskop fluorescence microscope (magnification, ×1,000; Carl Zeiss, Germany). Images were obtained with a Canon Powershot G3 digital camera.

Fatty acid analysis. Cultures (1 liter) of mid-exponential-phase cells in BHI were left untreated or treated with BC (2 $\mu g\ ml^{-1}$) for 30 min and subsequently centrifuged (Beckman Avanti J-25; Beckman Coulter Inc.). The obtained pellets were washed twice with 50 ml sterile Milli-Q water. In order to extract the cell lipids, the method of Bligh and Dyer (8) was used. Briefly, a monophasic system was obtained by adding 3.75 ml of a methanol-chloroform (2:1, vol/vol) solution for each ml of bacterial suspension. Samples were kept at room temperature for 1 h shaking at 200 rpm. In order to convert the system into a diphasic state, 2.5 ml of a chloroform–Milli-Q water (1:1, vol/vol) mixture was added for each ml of bacterial suspension and samples were subsequently incubated at room temperature for 1 h shaking at 200 rpm. Afterwards, samples were centrifuged at 1,000 × *g* for 5 min (Centrifuge 5804R; Eppendorf) and the hydrophobic phase, containing the cellular lipids, was collected. Subsequently, the solvent was evaporated and the lipid residue was immediately dissolved in hexane (VWR International, West Chester, PA) and stored at −20°C. To obtain the fatty acid methyl esters (FAME), an alkaline medium containing $NaOCH_3$ was used as described elsewhere (41). The membrane fatty acid composition was determined on a gas chromatographer (Trace GC; ThermoFinnigan, CA). Fatty acids were identified by comparing their retention times with those of known standards (bacterial acid methyl ester; Supelco, Sigma), and results were analyzed using the Xcalibur software (version 2.0.0.0; ThermoFinnigan). The relative percentage of each fatty acid was calculated as the ratio of the surface area of the considered peak to the total area of all peaks.

Oxidation of sulfhydryl groups. To analyze the extent of oxidation of sulfhydryl groups induced by disinfectants, total protein samples were isolated after 60 min after the addition of disinfectants. Cell-free protein extracts were obtained from 50-ml *B. cereus* cultures by using a method described elsewhere (45). The measurement of free-sulfhydryl groups was carried out, using a colorimetric reagent that turns yellow upon reaction with the reduced-sulfhydryl groups, as previously described (21). Briefly, a 10 mM stock solution of 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) (Sigma) was prepared in 0.1 M PBS (pH 7.0) and stored at −20°C. The A_{412} was adjusted to zero by using samples diluted in 0.1 M PBS (pH 8.0). To measure the amount of sulfhydryl groups, the DNTB reagent was added to the protein samples to a final concentration of 0.6 mM and the A_{412} was measured after 5 min incubation. The number of free-sulfhydryl groups in each sample was calculated according to the formula $C_0 = (A/\varepsilon \cdot l) \cdot D$, with A corresponding to the A_{412}, ε to the extinction coefficient (13,600 $M^{-1}\ cm^{-1}$), l to the path length (0.5 cm), and D to the dilution factor. As a control, protein samples extracted from untreated cultures were used. Protein concentrations were determined by the bicinchoninic acid (BCA) assay (55), with bovine serum albumin (BSA) (Sigma-Aldrich) as a standard. The free-sulfhydryl content was expressed as mmol per g of total protein extract.

Mutation rate. In order to investigate mutation rates upon exposure to disinfectants, the rifampin resistance (Rif^r) of exposed cultures was evaluated. Resistance to rifampin can be caused by mutations within the *rpoB* gene and can be used to evaluate the mutation frequencies of bacterial cultures (65). Exponentially growing cultures (100 ml) were treated with mild, growth-arresting, or lethal concentrations of BC, SH, HP, or PAA. At 0, 10, 30, and 60 min after disinfectant treatments, 20 ml of the cultures were spun down (11,000 rpm for 30 s) and the pellets were resuspended in 1 ml PBS, concentrating the cell suspensions. Cell suspensions and corresponding dilutions in PPS were plated on BHI agar plates containing 50 $\mu g\ ml^{-1}$ rifampin (Sigma) for detection of Rif^r colonies and on BHI agar plates without antibiotics for determination of the viable cell titers. Colonies were counted after 24 and 48 h of incubation at 30°C. Mutation frequencies were calculated as mutants per total viable cell count, and the mutation rate was calculated as the ratio between mutation frequencies at t_x and t_0.

Microarray data accession number. The microarray data from this study have been deposited in the GEO database (GEO accession number GSE18807).

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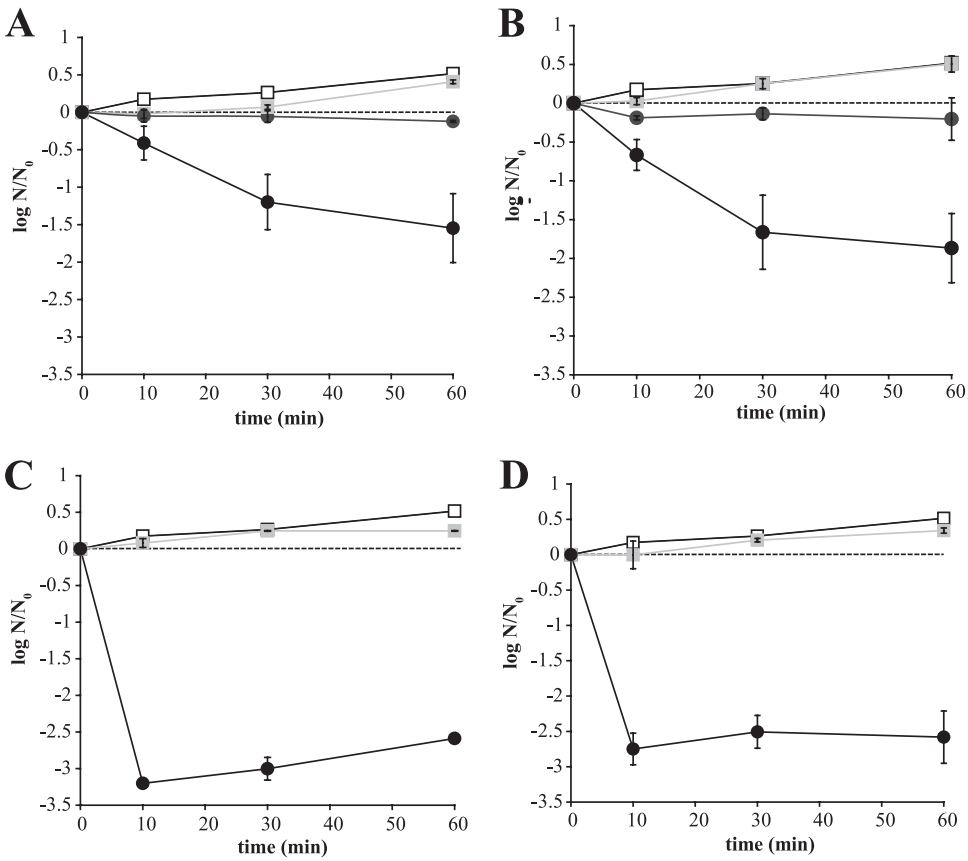

FIG. 1. Response of mid-exponential-phase (OD_{600}, ~0.5) *B. cereus* ATCC 14579 cells upon exposure to BC (A), SH (B), HP (C), and PAA (D) treatments. For each compound, different concentrations were selected, i.e., mild concentrations (1 μg ml^{-1} BC; 300 μg ml^{-1} SH; 0.05 mM HP; 10 μg ml^{-1} PAA) (light gray squares), growth-arresting concentrations (2 μg ml^{-1} BC; 400 μg ml^{-1} SH) (dark gray circles), and lethal concentrations (5 μg ml^{-1} BC; 500 μg ml^{-1} SH; 0.2 mM HP; 100 μg ml^{-1} PAA) (black circles). As a reference, cell counts of untreated *B. cereus* ATCC 14579 cultures are shown (open squares). The dashed line represents the initial level of CFU. The averages of two independent experiments are shown as $\log(N/N_0)$, and error bars indicate standard deviations.

RESULTS

Stress selection. To investigate the effect of disinfectant exposure on *B. cereus* growth, dose-response studies were conducted, treating mid-exponential-phase cells (OD_{600}, ~0.5) with a broad range of concentrations of BC, SH, HP, and PAA (see Fig. S1 in the supplemental material). Concentrations of BC, SH, HP, and PAA equal to or lower than 2 μg ml^{-1}, 400 μg ml^{-1}, 0.07 mM, and 10 μg ml^{-1}, respectively, resulted in attenuation or arrest of cell growth, while higher disinfectant concentrations led to inactivation of *B. cereus* cells.

Concentrations leading to different phenotypic responses, i.e., attenuation of cell growth, growth arrest for SH and BC, and 2 to 3 log reductions of viable cell counts, were selected (Fig. 1) and used throughout this study. Growth-arresting concentrations of HP and PAA were not identified. Therefore, only attenuating and lethal concentrations of HP and PAA were used in this study. Furthermore, exposure to the lethal concentrations of HP (0.2 mM) and PAA (100 μg ml^{-1}) did not lead to a time-dependent inactivation of the cells, as observed for exposure to lethal levels of BC and SH. The 3-log reduction achieved after lethal HP or PAA exposures was observed within 2 min of exposure (data not shown).

General transcriptome response to disinfectants. DNA microarrays were used to analyze gene expression patterns in *B. cereus* ATCC 14579 cells after exposure to SH, BC, HP, and PAA. For each stress condition, samples were collected after 10 and 30 min exposure and compared with untreated samples. To compare conditions, a hierarchical cluster analysis (Pearson correlation, average linkage) was performed on the expression profiles resulting from SH, BC, HP, and PAA treatments. The analysis showed a separation of the samples into three main branches (Fig. 2), clearly correlating with the disinfectant used: the upper branch included all the HP- and PAA-treated samples, the intermediate branch included the SH-treated samples with the exception of the sample treated with mild concentration of SH for 30 min, and all the BC-treated samples belonged to the lower branch. Furthermore, for almost all conditions tested, disinfectant treatments leading to a similar physiological response (mild, growth-arresting, or lethal treatments) clustered together independently of the exposure time. This analysis indicates that exposure to disinfectants leads to disinfectant-specific transcriptome responses. However, commonalities were also found between the different disinfection exposures. Genes with similar expression

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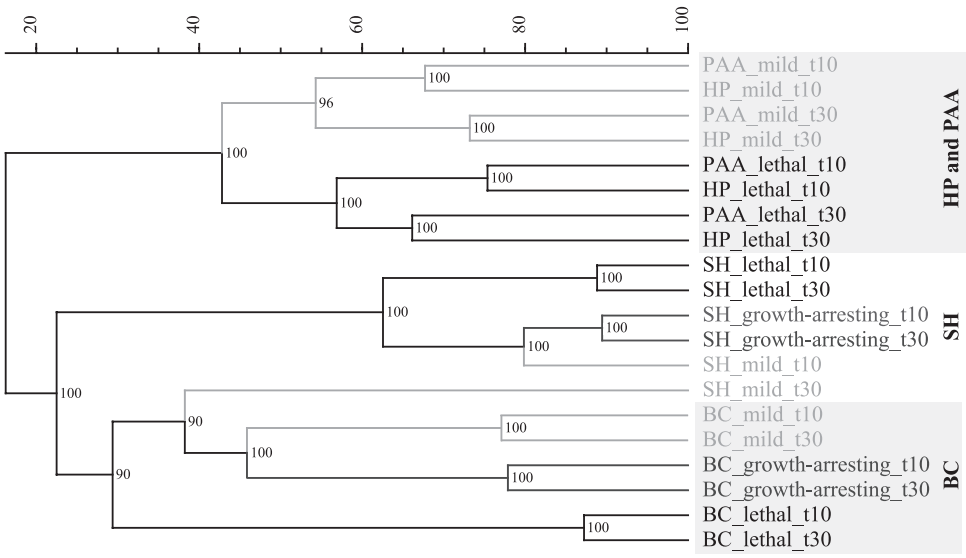

FIG. 2. Dendrogram of hierarchical clustering of expression profiles of disinfectant-stressed *B. cereus* cultures. Similarity was calculated using Pearson correlation and average linkage. For the calculation, all the genes represented on the microarray (5,352) were used. Samples obtained from cultures treated with lethal concentrations of disinfectants are depicted in black, samples obtained from cultures treated with growth-arresting concentrations are depicted in dark gray, and samples obtained from cultures treated with mild concentrations are depicted in light gray. Relative distance similarity between the branches is shown at the top, and bootstrap values are indicated at each branch.

changes in response to the different disinfectants were grouped and organized into different clusters by using hierarchical clustering (Euclidean distance, complete linkage). Two of these clusters, containing genes that were commonly differently expressed (up- or downregulated) upon exposure to mild and severe disinfectant treatments, are discussed in more detail in the supplemental material (see Tables S1 and S2). Genes belonging to the selected clusters were grouped into functional classes (Fig. 3). Of 65 annotated genes that were downregulated upon exposure to the different concentrations of disinfectants, 29% were involved in energy production and conversion. Six of these downregulated genes (BC2118, BC2119, BC2120, BC2121, BC2136, and BC3439) coded for proteins involved in the reduction of nitrogen compounds (nitrate and hydroxylamine) to ammonia, and four genes (BC1938, BC1939, BC4792, and BC4793) coded for subunits of the cytochrome *bd* complex, a terminal oxidase in the electron chain (16). Of 90 annotated genes commonly upregulated, 24% and 13% encoded proteins involved in inorganic ion transport and metabolism and proteins involved in posttranslational modification, protein turnover, and chaperones, such as *groES* (BC0294) and *groEL* (BC0295), respectively. Among the genes that were commonly upregulated, genes known to be involved in the general stress response, such as proteases (*clpC* [BC0100], *clpB* [BC1168], BC2793, BC5152, and BC0862) and multidrug resistance proteins (BC1786, BC2310, and BC5182), were also present. In addition, expression of the gene encoding alternative transcription factor σ^B (*sigB* [BC1004]), which is involved in the adaptation to stress (61), was generally upregulated. This finding suggested the involvement of σ^B in the stress response of *B. cereus* ATCC 14579 induced by disinfectants. However, exposure of *B. cereus* ATCC 14579 *rsbY* (60) and *rsbK* (17) deletion mutants, which are unable to activate SigB, showed no sensitization toward lethal concentrations of each disinfectant (data not shown).

Oxidative stress caused by disinfectant treatments. Oxidative stress is commonly induced by chemicals (67). Furthermore, exposure of aerobically growing *B. cereus* cells to preservation stresses has been shown to induce secondary oxidative stress (39, 40). Therefore, we focused on genes related to oxidative stress that were differently expressed after exposure to the selected disinfectants. A total of 15 genes putatively involved in oxidative stress were selected using the Oxygene Web-based program (58), and additionally 13 genes, whose orthologue genes were involved in oxidative stress response of *Bacillus subtilis* during vegetative growth (67), were selected. The analysis of these 28 genes revealed that up to 19 genes were upregulated after exposure to disinfectants (see Table S3 in the supplemental material). Notably, oxidative stress response-related genes (e.g., that for superoxide dismutase [BC4272]) were highly induced after exposure to growth-arresting concentrations of SH and BC (19 and 18 upregulated genes at at least one time point, respectively) and lethal concentrations of HP and PAA (18 and 16 upregulated genes at at least one time point, respectively) (Fig. 4).

Upregulation of the oxidative stress-related regulators such as the peroxide operon regulator (*perR* [BC0581]), Spx (BC3402), and OhrR regulator (BC4474) occurred in many conditions analyzed; in particular, upregulation of *perR* was observed when cells were treated with growth-arresting or lethal concentrations of disinfectants. The gene encoding regulator OhrR was upregulated after SH and PAA treatments and after lethal HP treatment, while BC treatment did not affect the transcription of this regulator. Two Spx-type regulators could be identified in the genome of *B. cereus* ATCC 14579; BC1188 was upregulated in some conditions, while BC3402

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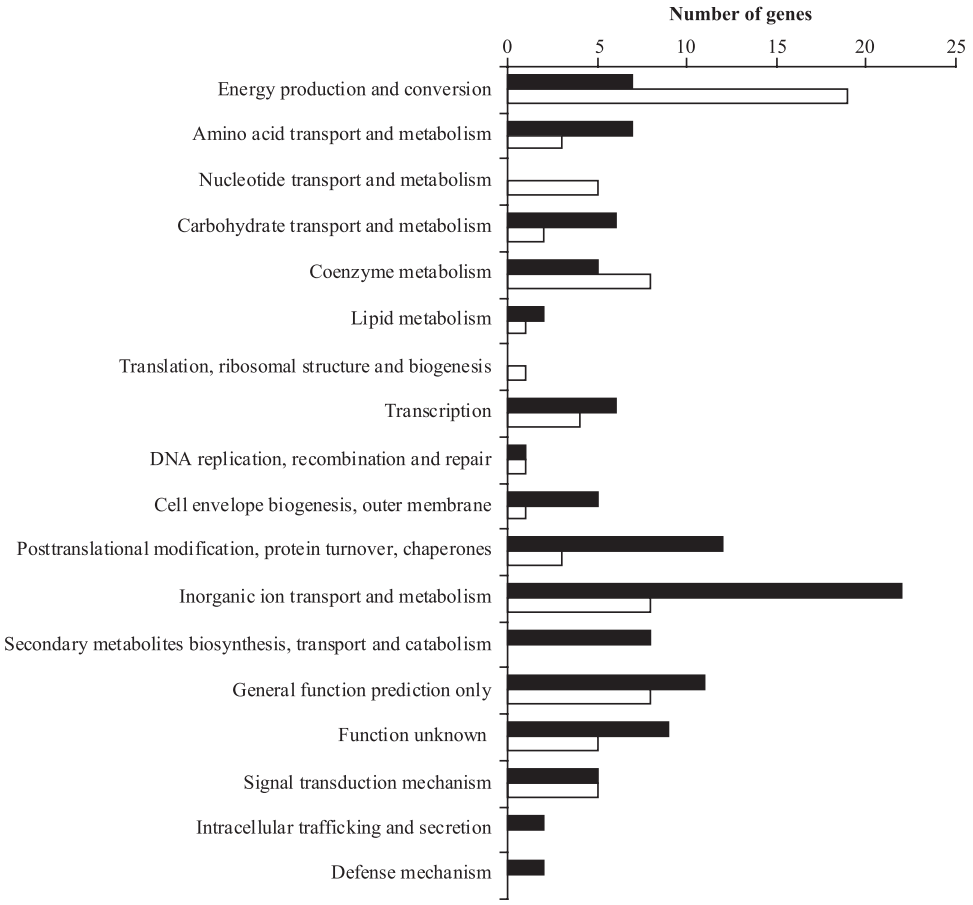

FIG. 3. Numbers of genes belonging to the clusters containing commonly upregulated (black bars) or downregulated (white bars) genes upon exposure to the different disinfectant treatments classified using COG functional categories (from the NCBI database: http://www.ncbi.nlm.nih.gov/COG/).

was commonly upregulated. Moreover, genes primarily involved in oxidative stress, such as those encoding catalases (*katE* [BC0863] and *katA* [BC1155]), thioredoxin reductases (BC0385, BC5159), and the alkyl hydroperoxide reductase *ahpCF*, described as a scavenger of endogenous hydrogen peroxide in *Escherichia coli* (51), were upregulated in almost all conditions analyzed.

Benzalkonium chloride-induced responses. Exposure to BC activated transcription of genes encoding QAC resistance proteins, and a similar response was observed in cells exposed to growth-arresting and lethal concentrations of SH.

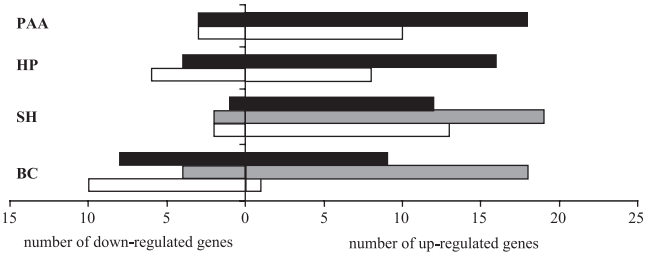

FIG. 4. Genes involved in oxidative stress response differently expressed at at least one time point in *B. cereus* ATCC 14579 after mild (white bars), growth-arresting (gray bars), and lethal (black bars) disinfectant exposure.

More specifically, genes putatively involved in fatty acid metabolism were upregulated when *B. cereus* cells were treated with growth-arresting and lethal concentrations of BC. In particular, genes coding for proteins such as acetyl coenzyme A (acetyl-CoA) acetyltransferase (BC4023, BC5003) and enoyl-CoA hydratase (BC0898, BC4524, BC5004), involved in the first steps of the β-oxidation of fatty acids (1), were upregulated. This result may correlate with the mode of action of BC, i.e., the ability to cause membrane damage. Disinfectant-induced membrane damage was assessed using lethal concentrations of the four disinfectants (5 μg ml^{-1} BC, 500 μg ml^{-1} SH, 0.2 mM HP, and 100 μg ml^{-1} PAA). Unstressed cells and cells treated for 10 and 30 min with disinfectants were stained with the membrane-permeant green fluorescent DNA probe SYTO-9 and the red fluorescent probe PI, a DNA stain that permeates only cells with compromised membranes. The results obtained confirmed the ability of BC to cause membrane damage, as the BC-treated sample exhibited red fluorescent cells within 10 min of treatment. No red cells were observed, at least within 30 min exposure, when samples were treated with lethal levels of SH, HP, and PAA (see Fig. S2 in the supple-

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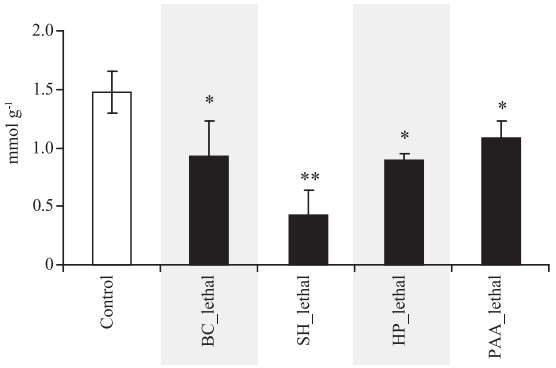

FIG. 5. Concentration of free-sulfhydryl groups (mmol g^{-1} of total protein) in cell extracts after 60 min exposure to the lethal concentration of disinfectants (BC, SH, HP, and PAA). Error bars represent the standard deviations of results for three averaged replicates. *, significantly different from value for control conditions; **, significantly different from values for all other conditions ($P \leq 0.05$, Student's *t* test).

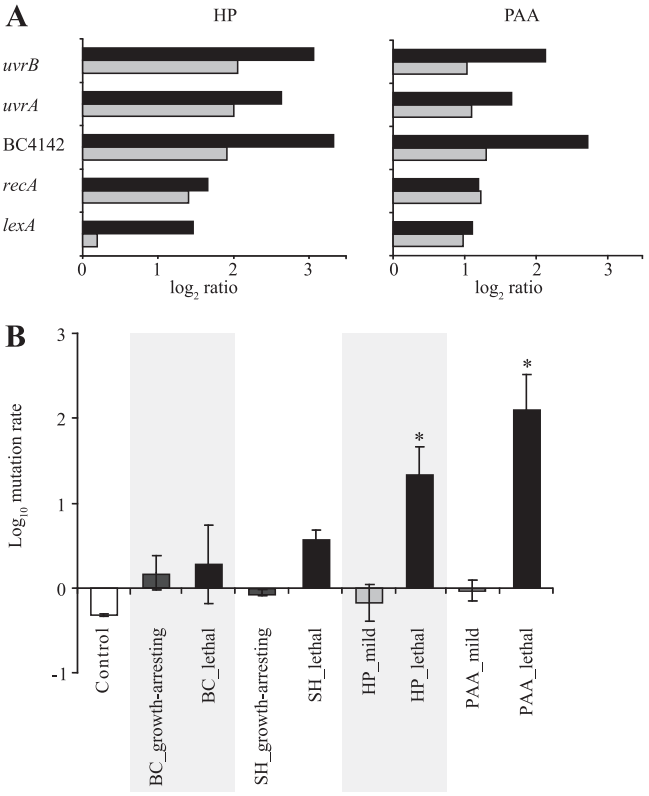

FIG. 6. (A) Expression of genes putatively involved in the SOS response of *B. cereus* after mild (light gray bars) and lethal (black bars) HP and PAA treatments. (B) Log_{10} mutation rates of *B. cereus* ATCC 14579 after 60 min exposure to mild (light gray bars), growth-arresting (dark gray bars), or lethal (black bars) concentrations of disinfectants (BC, SH, HP, and PAA). Results obtained using an untreated sample are depicted with a white bar. Error bars represent the standard deviations of the averages. Asterisks (*) denote statistical significance ($P \leq 0.05$, Student's *t* test).

mental material). Furthermore, the fatty acid composition of cells exposed to a growth-arresting concentration of BC was compared to that of untreated cells. This analysis showed that the fatty acid composition of *B. cereus* ATCC 14579 was modulated such that in BC-treated cells, fatty acids with shorter carbon chains were overrepresented compared to the ones with longer chains (data not shown). A preferential increase in short-chain fatty acids was previously observed in UV- and carvacrol-stressed *B. cereus* cells (5, 59).

Sodium hypochlorite-specific responses. The transcriptome analyses of cells treated with SH revealed a strong upregulation of transcripts encoding oligopeptide binding proteins (BC0207-B0210, BC0215-B0216, and BC1180-B1183), independently of the concentration of SH used. SH treatments also resulted in the upregulation of genes putatively involved in organic sulfur transport and metabolism, which has been observed previously in *Pseudomonas aeruginosa* (54). In the present study, genes coding for proteins involved in the reduction of sulfate to sulfite (BC1421-BC1423) were induced in *B. cereus* cells upon treatment with SH, while no differential expression of these genes was observed upon exposure to the other disinfectants used. Reduction of sulfate is one of the first steps in the biosynthesis of sulfur-containing amino acids, such as cysteine and methionine (52). Upregulation of genes involved in cysteine and methionine biosynthesis pathways was also observed after exposure to SH. Hypochlorous acid, which is formed out of SH in aqueous environments, has been described to cause oxidation of cysteine-associated sulfhydryl groups (47). The oxidation of sulfhydryl groups conceivably triggers the induction of sulfur and sulfur-containing amino acid pathways. Therefore, the free-sulfhydryl content of cultures treated with lethal concentrations of the four disinfectants (5 μg ml^{-1} BC, 500 μg ml^{-1} SH, 0.2 mM HP, or 100 μg ml^{-1} PAA) was measured. Cell extracts, obtained from cultures treated with disinfectants for 60 min and from untreated control cultures, were investigated. Based on the reduced color reaction, a significant decrease of the free-sulfhydryl content was apparent in the cells treated with all the different disinfectants (Fig. 5). However, treatment with a lethal concentration of SH resulted in a more prominent oxidation of sulfhydryl groups compared to the oxidation induced by BC, HP, or PAA.

Hydrogen peroxide- and peracetic acid-specific responses. A large overlap between the HP- and PAA-induced transcriptome responses was observed (Fig. 2). Eighty-five percent of the genes differently expressed after mild and lethal PAA exposure were also differently expressed in cells exposed to mild and lethal concentrations of HP. The remaining 15% of genes showed similar expression between the HP- and PAA-treated cultures; however, this expression was not the same at all time points or concentrations used. Therefore, the responses of *B. cereus* cells to HP and PAA are described in this section together. The genes of the linear plasmid of *B. cereus* ATCC 14579 were among the most highly upregulated genes upon exposure to both HP and PAA. Furthermore, DNA repair-related genes were specifically induced after HP and PAA treatments, in particular genes putatively involved in the SOS response, such as *recA* and *lexA* (Fig. 6A). Similar trends were observed for exonuclease ABC subunit-encoding genes *uvrA* and *uvrB* (BC5167, BC5168), which are putatively involved in the excision of nucleotide fragments during DNA repair (34). Among the genes that were upregulated, a gene coding for a DinB-like DNA polymerase IV (BC4142) was present. Such

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DNA polymerases have been described as error-prone polymerases in *E. coli* (23). Therefore, the effect of disinfectant treatments on the mutation rate was investigated. Mid-exponential-phase *B. cereus* ATCC 14579 cultures, treated with different concentrations of disinfectants, were tested for rifampin resistance, and the mutation rate was calculated after 10, 30, and 60 min of exposure. Similar mutation rates were obtained at 10, 30, and 60 min of exposure; therefore, only the results obtained after 60 min are shown (Fig. 6B). The results obtained showed that the lower concentrations of disinfectants did not significantly increase the mutation rate compared to that of the unstressed control cultures. Similar results were obtained after treatments with lethal concentrations of BC and SH. Only the treatments with lethal concentrations of HP and PAA resulted in a significantly higher mutation rate than in the untreated cultures.

DISCUSSION

Nowadays, disinfectants are commonly applied in food industries (30). Nevertheless, little information is available on the molecular mechanisms displayed by microorganisms in response to disinfectants. Understanding the molecular responses induced by these compounds is essential in order to comprehend how bacteria adapt to food-processing environments, including the generation of resistant variants. In this study, the whole-genome expression profiles of *B. cereus* ATCC 14579 in response to four disinfectants, i.e., benzalkonium chloride (BC), sodium hypochlorite (SH), hydrogen peroxide (HP), and peracetic acid (PAA), were determined over an interval of 30 min. For each compound, concentrations leading to attenuation of bacterial growth, growth arrest, or cellular inactivation were tested to obtain comprehensive insights into the response of *B. cereus* upon exposure to disinfectant treatments. Moreover, investigations on diverse phenotypic parameters were performed and data were correlated with the responses observed at the transcriptome level.

Systematic examination of the global transcriptional response of *B. cereus* to disinfectant treatments revealed that genes important for basal metabolism, such as genes related to nitrogen metabolism and oxidative phosphorylation, were repressed after different stresses (Fig. 3). This feature of the transcriptional response to stresses might reflect a general decrease of the cellular growth rate in stressed cells, rather than a direct stress-mediated repression. Furthermore, comparative analysis of the transcriptional responses induced by disinfectants revealed a common upregulation of stress-related genes. In particular, the transcript of the σ^B-encoding gene was upregulated. This finding suggested a role for σ^B during disinfectant exposure in *B. cereus*, corroborated by the previously described induction following different stress responses in many bacteria (11, 60), as well as in the resistance of *Listeria monocytogenes* to detergents such as QAC (48). However, in the current study, the deletion of the *rsbY* and *rsbK* genes, encoding proteins involved in the activation of σ^B (17, 61), did not sensitize *B. cereus* ATCC 14579 to disinfectant stresses. This result suggests that σ^B, even though induced, has no direct role in protecting *B. cereus* cells against disinfectant treatments. Nevertheless, the increased expression of *sigB* may result in increased resistance to subsequent stresses, such as heat treatments (61), that cells may experience after recontamination of food and further processing. Alternatively, other cellular defense systems may have been activated in these mutants. Indeed, other regulators, such as the ones involved in oxidative stress response, could play a more prominent role in the response of *B. cereus* to disinfectant treatments. This study revealed that the *perR* regulator gene and several other genes known to be involved in oxidative stress response (67) were induced. Moreover, as shown for salt-stressed *B. cereus* cells, the activation of the oxidative stress response and, in particular, the increased catalase activity can lead to possible cross-protection mechanisms that may contribute to the higher resistance of cells to different stresses (19).

Together with the activation of oxidative stress-related genes, increased expression of genes coding for several ion transporters was observed (Fig. 3). Intracellular free-iron levels may have been affected by the exposure to oxidative stress (26). In fact, oxidative stress can cause liberation of iron from proteins harboring Fe-S centers (25). The intracellular iron concentration is a critical parameter for cell survival, as free iron can react with HP, forming hydroxyl radicals in the Fenton reaction (27). Transcriptome studies on the response of *Staphylococcus aureus* to HP and PAA showed that iron uptake systems were initially repressed (12, 14), suggesting that this bacterium modulates iron uptake in order to prevent secondary oxidative damage. On the other hand, it has also been demonstrated that increased levels of intracellular HP can cause *Escherichia coli* cells to misperceive the real concentration of iron by converting the ferrous iron into ferric iron, which subsequently leads to a dramatic upregulation of iron import systems (63). The induction of iron uptake systems in *B. cereus* upon disinfectant exposure suggests that similar deregulation of the intracellular iron homeostasis may occur. In addition, upregulation of other putative divalent cation transporters may stimulate accumulation of, e.g., magnesium and manganese, which may directly or indirectly contribute to oxidative stress resistance by replacing iron in catalytic sites of specific enzymes and/or oxygen radical scavenging by manganese (37).

In this study, exposure to BC resulted in upregulation of genes involved in fatty acid metabolism. Similar results were obtained when *Saccharomyces cerevisiae* cells were treated with detergents, such as sodium *n*-dodecyl benzene sulfonate and sodium dodecyl sulfate (53). This result is in accordance with the ability of BC to cause membrane damage (see Fig. S2 in the supplemental material) and modification of the composition of the cell membrane. Modulation of fatty acid composition has been shown before for *B. cereus* exposed to irradiation (5). Exposure of *B. cereus* cells to BC and SH treatments (see below) also resulted in upregulation of QAC resistance proteins. These membrane-bound proteins are able to extrude several antimicrobial compounds (33), and moreover, their increased activity may enhance bacterial tolerance toward other antibacterial compounds (4, 7, 46).

SH exposure of *B. cereus* ATCC 14579 resulted in an increase of transcripts involved in sulfur metabolism, including anabolic pathways for sulfur-containing amino acids, such as cysteine and methionine, as well as peptide transporters that may supply the cell with peptides containing these amino acids and/or their precursors. Some of these responses have been

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observed in SH-treated *P. aeruginosa* (54) and in *B. subtilis* cells exposed to the O_2^--generating agent paraquat (42). The induction of sulfur-related genes upon exposure to SH correlates with the finding that exposure to SH showed a significantly higher oxidation of sulfhydryl groups in *B. cereus* cells (Fig. 5). This is in accordance with the notion that strong oxidizers are known to react with biological substrates containing sulfhydryl groups (2). The ability of SH to oxidize sulfhydryl groups could lead to inhibition of enzyme activity (6), finally leading to failure of cell functioning.

Exposure of *B. cereus* ATCC 14579 to HP and PAA resulted in increased levels of genes coding for error-prone DNA repair mechanisms, including the SOS response. The SOS response is an inducible DNA repair system that can provoke error-prone repair and facilitate the emergence of mutations (28). Notably, exposure of *P. aeruginosa* to PAA did not result in upregulation of genes involved in the SOS response (13), whereas responses linked to DNA damage repair have been noted in other bacteria treated with HP (12, 42, 44). Activation of the SOS response, including the activity of the RecA protein, can also trigger inactivation of phage repressors inducing the lytic cycle of prophages (22). This is in accordance with the specific HP- and PAA-induced upregulation of the entire set of genes located on the *B. cereus* ATCC 14579 linear plasmid pBClin15, which is probably a prophage (57, 64). Furthermore, our data showed that treatments with lethal HP and PAA concentrations resulted in significantly increased mutation rates in *B. cereus* cells (Fig. 6B). Increased mutation rates may contribute to the genotypic heterogeneity of these disinfectant-treated cultures. The impact of HP and PAA exposure on cross-protection of *B. cereus* to other stresses and on the generation of stress-resistant variants remains to be elucidated.

In conclusion, exposure of aerobically grown *B. cereus* cells to a selection of widely used disinfectants resulted in an extensive oxidative stress response, indicating generation of reactive oxygen species and perturbation of cellular redox balance. Furthermore, the different disinfectant treatments corresponded to specific transcriptional and physiological responses, indicating that different vital cellular processes may be targeted. These findings could suggest that combinations of disinfectants may have synergistic effects in killing pathogens and may aid in selecting optimal combinations of disinfectants and/or in designing more efficient sequential cleaning procedures. In addition, the combined transcriptome and phenotype analysis revealed activation of DNA damage repair systems, such as the SOS response, that may contribute to the generation of genetic heterogeneity and occurrence of stress-resistant variants. These phenomena may affect the recontamination capacity of *B. cereus* and, thus, food quality and safety.

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