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Toxicogenomic Response of *Staphylococcus aureus* to Peracetic Acid

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Staphylococcus aureus is responsible for many incidents of hospital-acquired infection, which causes 90 000 deaths and $4.5 billion loss a year in the United States. Despite a wide use of disinfectants such as peracetic acid in health care environments, we certainly need better understanding of the effects of antimicrobial application on target pathogens to avert infection outbreaks. Consequently, herein, we explored for the first time the toxicogenomic response of *S. aureus* to a sublethal concentration of peracetic acid (1 mM) by using microarray-based transcriptome analysis. In particular, we investigated the dynamics of global gene expression profiles during its cellular response, which involved initial growth inhibition (10 min) and subsequent partial recovery (20 min). Further, we compared transcriptome responses to peracetic acid between *S. aureus* and *Pseudomonas aeruginosa*. Our findings show that (i) the regulation of membrane transport genes was significantly altered, (ii) DNA repair and replication genes were selectively induced, and (iii) primary metabolism-related genes were differently repressed between the two growth states. Most intriguingly, we revealed that many virulence factor genes were induced upon the exposure, which proposes a possibility that the pathogenesis of *S. aureus* may be stimulated in response to peracetic acid.

Introduction

The U.S. Environmental Protection Agency (EPA) has endeavored to determine the efficacy and the mode of action of antimicrobials. At EPA, 5000 antimicrobial products are registered, and hospital-level disinfectants are being tested against pathogens such as *Staphylococcus aureus*, which is responsible for many infectious diseases, ranging from benign skin infections to life-threatening endocarditis and toxic shock syndrome (*1*). One of the reasons EPA has exerted such efforts is that hospital-acquired infections are a serious threat to public health. Every year, in the United States alone, 2 million patients get an infection during hospital treatment, and 90 000 die as a direct or indirect result of these infections (*2*). Furthermore, economic damage associated with the hospital infection is estimated to be nearly $4.5 billion a year (*2*). Therefore, it is extremely crucial to use appropriate antimicrobial agents with a clear understanding of the subsequent effects to prevent infection outbreaks in health care environments (*3*).

Oxidative antimicrobials including peracetic acid and hydrogen peroxide have been widely used to kill pathogens including *S. aureus* in hospital environments. Peracetic acid, combined with hydrogen peroxide, is an active ingredient of EPA-registered disinfectants because of its high antimicrobial efficacy and nontoxic residuals. Thus, EPA has investigated the antimicrobial activity of peracetic acid to understand how to apply it more effectively. However, even though the activity speculatively involves the release of active oxygen, we still have many unanswered aspects of the mechanism of action (*4*). Moreover, a lack of understanding of a cellular response to peracetic acid hinders further development of more innovative methods for combating pathogens. Certainly, better elucidation of the molecular events responsible for establishing and maintaining pathogenicity will help to map affected cell functions and serve to delineate the mechanisms involved in the disinfectant activity.

Microarrays have been effectively employed to simultaneously and globally examine the complete transcriptional response at the genomic level in *Escherichia coli*, *Pseudomonas aeruginosa*, and *S. aureus* upon exposure to antimicrobials (*5*−*9*). Furthermore, in our previous study, we explored the response of *P. aeruginosa* to peracetic acid by employing array-based transcriptome analysis (*7*). Herein, to widen our knowledge of the mechanisms involved in the antimicrobial activity of peracetic acid, we investigated *S. aureus*, another major pathogen of concern for human health at EPA. In particular, we studied the dynamics of global gene expression profiles during the cellular response of *S. aureus* to a sublethal concentration of peracetic acid (1 mM), which involved initial growth inhibition (10 min) and subsequent partial recovery (20 min), by utilizing Affymetrix *S. aureus* GeneChip arrays. We also performed real-time PCR analysis on selected genes to validate the array results. To our knowledge, this is the first study demonstrating the transcriptome analysis of *S. aureus* response to peracetic acid. Therefore, the outcomes presented here may enhance our understanding of the mechanisms involved in the toxicity of peracetic acid and inform future disinfectant protocols. Further, since peracetic acid also exists in the environment and is continuously formed through the oxidation of reactive organics in the atmosphere (*10, 11*), the results of this study may also have a potential impact on elucidating the interactions between peracetic acid and microbial ecosystems.

Materials and Methods

Affymetrix *S. aureus* GeneChip Analysis. In this study, we used *S. aureus* NCTC 8325 obtained from Network on Antimicrobial Resistance in *Staphylococcus aur*eus (NARSA). As previously described (*9*), we initiated and maintained *S. aureus* cultures at 37 °C with shaking at 250 rpm using sterilized Luria-Bertani (LB) broth. We added 1 mM peracetic acid (Aldrich Chemical Co., St. Louis, MO) immediately after the optical density at 600 nm reached the early logarithmic phase (0.8). Then, we isolated total RNA after 10 and 20 min incubation using the RiboPure − Bacteria kit (Ambion, Inc., Austin, TX) by following the manufacturer's protocol (*9*). Finally, we performed cDNA synthesis, labeling, hybridization, staining, and washing steps by following the manufacturer's protocol for the Affymetrix *S. aureus* GeneChip arrays (Affymetrix, Inc., Santa Clara, CA).

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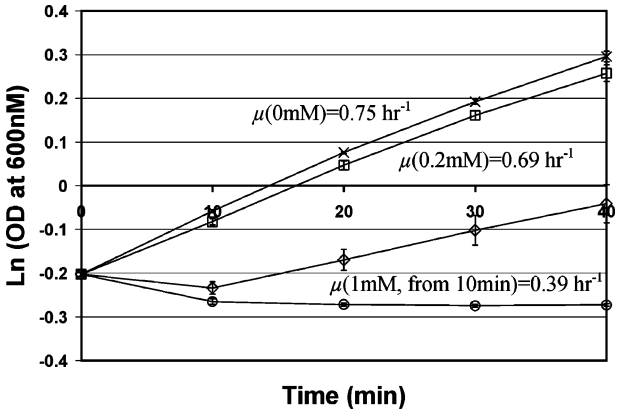

FIGURE 1. *S. aureus* growth (optical density at 600 nm) after treatment with peracetic acid: 0 mM (×), 0.2 mM (□), 1 mM (◇), and 5 mM (○). The growth rate (μ) was calculated during the exponential phase of the cells with 0 mM, 0.2 mM, and 1 mM peracetic acid. The results are the mean of triplicate experiments; the error bars represent standard deviation.

To analyze the array data, we utilized Affymetrix GeneChip Operating Software (GCOS) v. 1.2 and Data Mining Tool (DMT) v. 3.1 (Affymetrix, Inc., Santa Clara, CA) with the following parameters: *alpha* 1, 0.04; *alpha* 2, 0.06; *tau*, 0.015; target signal, 500. *Alpha* 1 and 2 are significance levels that define detection calls (see below), while *tau* determines analysis sensitivity (*12*). Further, the average intensity of arrays was scaled to a target signal. We calculated fold change as the ratio between the signal averages of three untreated (control) and three peracetic acid-treated (experimental) cultures for 10 and 20 min exposures. Gene expression fold changes were identified with statistical significance by a *t*-test (cutoff *p*-value, 0.05). The GCOS detection calls of "present", "marginal", and "absent" are determined based on the Affymetrix detection algorithm. This call indicates whether a transcript is reliably detected (present) or not detected (absent).

Real-Time PCR Analysis. To determine the validity of the array data, transcript level changes obtained with the microarray analysis were compared with those from quantitative real-time PCR. Genes and primer sequences employed for the real-time PCR analysis are listed in Supporting Information Table 1. The housekeeping gene 16S rRNA was used as an endogenous control (*9, 13*). We performed the real-time PCR by utilizing an iCycler iQ Real-Time PCR Detection System with an iScript cDNA Synthesis Kit and an IQ SYBR Green Supermix (BioRad Laboratories, Inc., Hercules, CA). For each gene, three biological replicates with three technical replicates each were employed. Reaction mixtures were initially incubated for 3 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, 30 s at 53.9 °C, and 20 s at 72 °C. PCR efficiencies were derived from standard curve slopes in the iCycler software v. 3.1 (BioRad Laboratories, Inc., Hercules, CA). Melt-curve analysis was also performed to evaluate PCR specificity and resulted in single primer-specific melting temperatures. In this report, relative quantification based on the relative expression of a target gene versus 16S rRNA gene was utilized to determine transcript level changes.

Results and Discussion

Growth Inhibition by Peracetic Acid. To determine the sublethal inhibitory effect of peracetic acid on *S. aureus*, we first exposed the exponentially growing cells to peracetic acid. In Figure 1, we demonstrate that 1 mM peracetic acid caused a growth arrest for about 10 min. Note that minimum inhibitory concentration of peracetic acid on *S. aureus* is reportedly 0.33 mM (*14*). Figure 1 shows that after this period, the treated cells partially recovered from the arrest, starting to grow at a constant rate, which is lower than that of the untreated cells. Thus, in this study, to better understand how *S. aureus* initially responds to peracetic acid and, subsequently, recuperate from the damage, we employed 10 and 20 min exposure times with 1 mM peracetic acid.

Microarray Analysis of *S. aureus* Response. To investigate the changes in transcriptome profiles in response to peracetic acid, we conducted three independent microarray experiments in the absence (control) and the presence (experimental) of 1 mM peracetic acid upon 10 and 20 min exposures. To further identify genes with statistically marked changes in expression levels, we applied the following criteria to each of the 10 and 20 min control-experimental microarray data sets: (i) a *p*-value for a *t*-test should be equal to or less than 0.05, (ii) an absolute fold change in transcript level should be equal to or greater than 2, and (iii) a gene should have a presence or marginal call (*12*) from 50% or more replicates on both the experimental and control replicate sets. Consequently, we found that 221 and 232 genes showed statistically marked increases and decreases in mRNA levels, respectively, after 10 min treatment. Upon 20 min exposure, 270 and 127 genes exhibited statistically marked expression level increases and decreases, respectively. Note that among these genes, 201 genes showed statistically marked changes upon both 10 and 20 min exposures. Therefore, 648 genes were differently expressed in response to either 10 or 20 min exposure. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) and are accessible through the GEO Series accession number GSE4184.

To test the validity of the microarray data, we further performed real-time PCR analysis on 7 genes with a range of fold changes (2−11-fold), which were specifically involved in the pathogenesis of *S. aureus* (discussed below). Supporting Information Table 1 shows that our microarray results were corroborated with real-time PCR analysis, which provides independent verification of transcript level changes of the genes that we discuss in this report.

Classification of Differently Regulated Genes. To assess how genes with transcript level changes are distributed with regard to their functions, we first classified the differently regulated 648 genes according to the categories described in the comprehensive microbial resource of the Institute of Genome Research (TIGR) (http:// www.tigr.org/tigr-scripts/CMR2/CMRHomePage.spl/). In Figure 2, we display the number of differentially regulated genes in each functional class. Figure 2 implies that the functional classes in general contained more induced and fewer repressed genes at 20 min. In particular, the functional classes of "cell envelope", "cellular processes", "hypothetical proteins", "regulatory functions", and "transport and binding proteins" had significantly more induced genes at 20 min. Further, fewer genes were found at 20 min in the classes of "cell envelope", "DNA metabolism", "energy metabolism", and "fatty acid and phospholipid metabolism". This result suggests that the functional class profiles were notably different between 10 and 20 min, and this difference might explain why *S. aureus* underwent the initial growth inhibition and the following partial growth recovery upon exposure to peracetic acid.

To further identify genes with similar transcription patterns during the time course, we categorized the 648 genes into 6 groups on the basis of their transcription directions. In Figure 3, we illustrated the expression patterns of genes of the groups, including their total gene numbers. Figure 4 displays the number of genes of Groups I−VI in each functional class. In Figure 4, a notable feature was that the expression of genes belonging to the class of "transport and binding proteins" was extensively altered upon the exposure, most of which are related to the active and/or facilitated

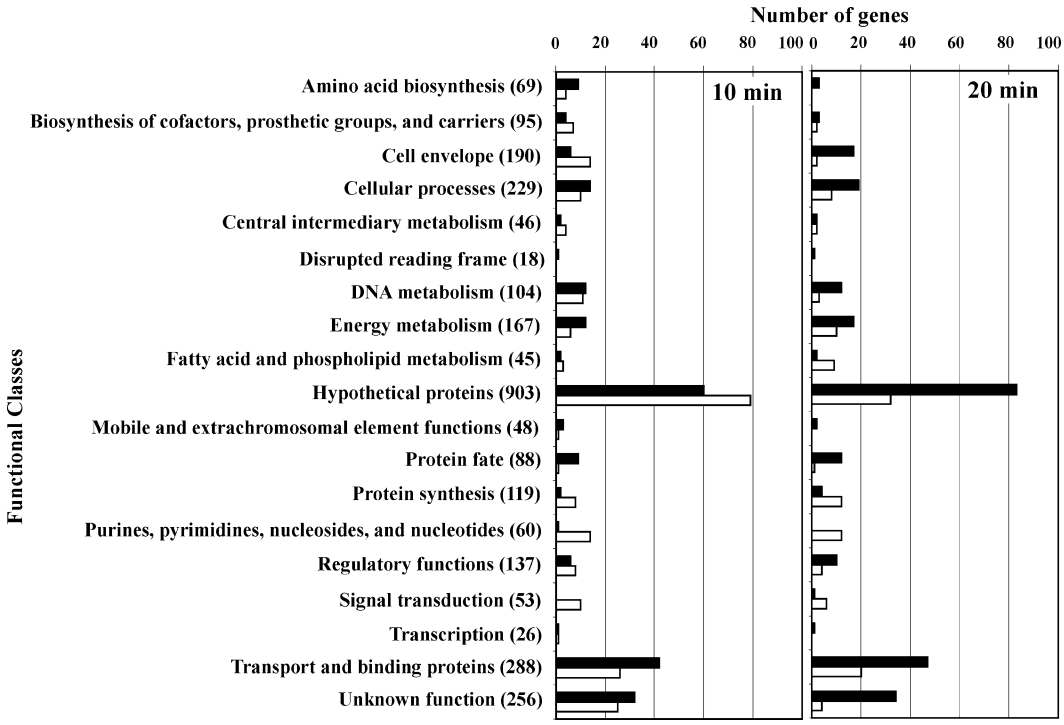

FIGURE 2. Functional classification of genes with statistically significant increase (■) and decrease (□) in mRNA level upon 10 and 20 min exposures (a total of 648 genes). The number in parentheses represents the total number of genes within the genome in each functional class.

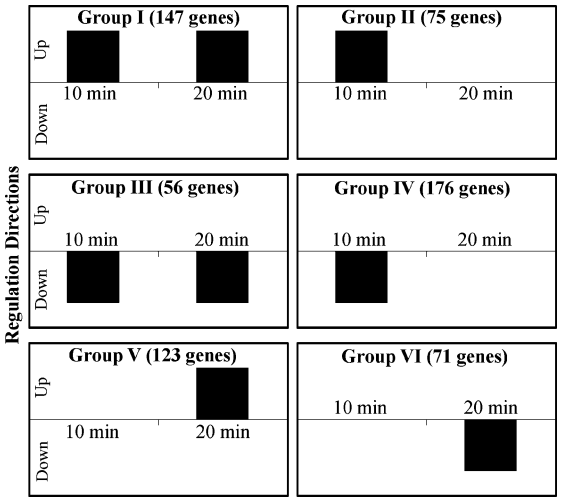

FIGURE 3. Groups of differentially regulated 648 genes categorized by their transcription directions upon 10 and 20 min exposures.

transport system in the membrane. Finally, Supporting Information Table 2 lists the genes of each group and their fold changes, *p*-values, and functional classes in response to 10 and 20 min exposures.

Group I: Genes Induced upon 10 and 20 Min Exposures. Figure 3 shows that Group I was composed of 147 genes that were upregulated upon both 10 and 20 min exposures. Figure 4 indicates that the most distinctive functional classes are "cellular processes", "DNA metabolism", "energy metabolism", "protein fate", and "transport and binding proteins". In particular, the class of "transport and binding proteins" constituted the largest portion of gene in Group I (27 genes). These genes primarily code for permeases and ATP-binding cassette (ABC) proteins. Supporting Information Table 2 shows that 15 genes encode ABC transporters carrying cations and iron, including SACOL0796-0797-0798, SACOL2165-2166-2167, and SACOL2721 (*nixA*). Further, SACOL0781 and SACOL0783 (*opuBB*) encode osmoprotectant ABC transporters, whereas SACOL1924 codes for a toxin-exporting ABC transporter. This result suggests that the regulation of genes related to membrane transport was altered upon exposure to peracetic acid.

Intriguingly, Supporting Information Table 2 shows that the majority of genes in the "cellular processes" class were associated with the pathogenesis of *S. aureus*. To be specific, SACOL0102 and SACOL0104 encode siderophore (iron-chelating compound) biosynthesis proteins, which enhance the virulence of *S. aureus* (*15*). SACOL0473 and SACOL1178 code for exotoxins, which are involved in food poisoning and toxic shock syndrome (*16*). Besides, alpha toxin encoded by SACOL1173 (*hlY*) is a pore-forming hymolytic toxin that causes membrane damage to many types of mammalian cells (*17*). SACOL0856 (*clfA*) codes for clumping factor A, which mediates the specific binding of *S. aureus* to fibrinogen and fibrin (*18*) Further, ClfA has a specific role as a virulence factor in the pathogenesis of staphylococcal endocarditis (*18*). This result suggests that the pathogenesis of *S. aureus* was indeed stimulated in response to peracetic acid, which might be used as one of the cellular protective mechanisms.

Supporting Information Table 2 also displays DNA repair-related genes in the class of "DNA metabolism". In detail, SACOL0823-0824 (*uvrAB*) and SACOL1157 (*uvrC*) encode enzymes mediating nucleotide excision repair (*19*), while SACOL1154 putatively encodes a DNA mismatch repair protein. SACOL1382 (*sbcC*) codes for a protein, which plays a role in eliminating long cruciform or palindromic sequences which would remove sequences that may interfere with DNA replication (*20*). The Nth protein (endonuclease III) encoded by SACOL1492 is a DNA glycosylase, involved in the first step of base excision repair of DNA damage in *E. coli* (*21*). The XerD protein encoded by SACOL1540 resolves chromosome dimers, which cannot be segregated to daughter cells during cell division (*22*) Last, COL-SA2131 encodes a Dps family protein, the DNA-binding ferritin-like protein, which plays a central role in protecting DNA from oxidative damage by directly binding to DNA (*23*). This result indicates that DNA

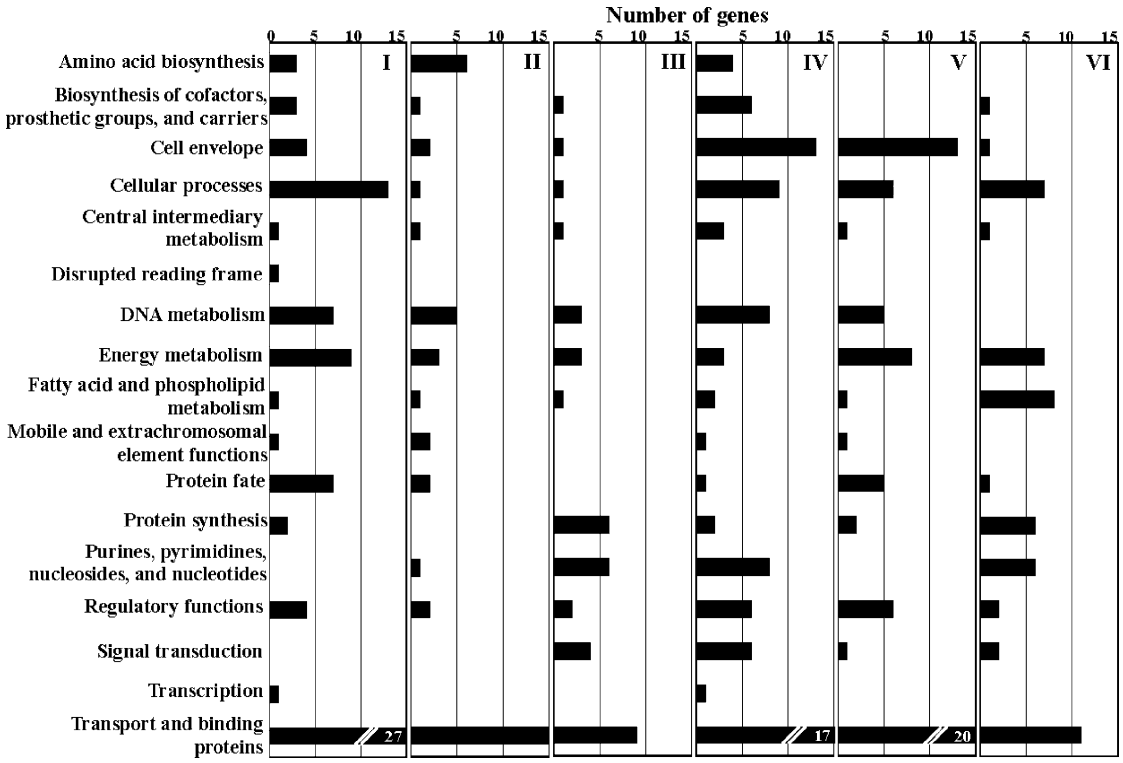

FIGURE 4. The number of genes within Groups I−VI in each functional class. Note that the functional classes of "hypothetical proteins" and "unknown function" are not included in this figure.

was damaged upon exposure to peracetic acid, concurrent with the previous outcome in *Pseudomonas aeruginosa* (*7*). More importantly, our data suggest that DNA repair system was continuously activated even after the growth of *S. aureus* resumed to some extent.

In relation to this result, we also found that bacterial competence genes were induced upon the exposure: SACOL0814, SACOL1004, SACOL1601, SACOL1644, SACOL1645, and SACOL1646 (*comEA*). Bacterial competence enables cells to bind and internalize exogenous DNA. More intriguingly, it was earlier hypothesized that competence serves as a function in DNA repair (*24*). Therefore, considering that DNA was likely damaged by peracetic acid (see above), we can speculate that the induction of the competence genes here is in line with the DNA repair hypothesis (*24*).

Of another importance was that SACOL1094-1095 (*cydAB*) was found in Group I. The *cydAB* genes together encode cytochrome *d* oxidase, which catalyzes the last step of oxygen respiration (*25*). Interestingly, it was revealed that cytochrome *d* oxidase plays an imperative part in cellular protection against oxidative stress (*26*). It was also suggested that the ability of cytochrome *d* oxidase to reduce dioxygen to water might minimize the generation of reactive oxygen species (*27*). Therefore, our result that the *cydAB* genes were strongly induced upon exposure to peracetic acid, which probably causes oxidative damage, strengthens the confidence of the prior assignments about the role of cytochrome *d* oxidase in oxidative protection processes.

As shown in Figure 4, genes in the "protein fate" class were distinctive in Group I as well as Group V. This finding is intriguing because the genes of the class in Group V, which exhibited upregulation after 20 min, code for proteases participating in protein repair and degradation, while the genes in Group I are mostly involved in protein secretion. In particular, SACOL0417-0418 encodes the proteins of the Mtt (membrane targeting and transport) system operon, which transport tightly folded or malfolded proteins to or across the membrane (*28*); and SACOL2672-2673-2674-2675 is also related to protein export, which may be organized in an operon. Last, SACOL0556 encodes a chaperone that preserves the integrity of essential intracellular proteins in response to environmental stressors such as oxidants (*29*).

Group II: Genes Induced upon 10 Min Exposure. Figure 3 shows that Group II comprised 75 genes with increased expression levels at 10 min; however, upon 20 min exposure, the expression level changes of these genes became statistically insignificant. First, Figure 4 shows that "transport and binding proteins" was also the most prominent functional class in Group II. In Supporting Information Table 2, the substrates of ABC transporters encoded by the genes of this class are primarily (i) amino acids, peptides, and amines (e.g. SACOL2411-2412) and (ii) cations and iron (e.g. SACOL0705-0706).

Interestingly, Figure 4 indicates that the class of "DNA metabolism", which had DNA repair genes, was conspicuous in this group as well. To be specific, SACOL1153 putatively codes for a DNA-dependent DNA polymerase family X protein, which is implicated in DNA repair processes in *Deinococcus radiodurans* (*30*) as well as eukaryotes (*31*). RecG encoded by SACOL1241 is involved in the repair of DNA damage resulting from quinolone treatment in *S. aureus* (*32*). The DnaD protein, putatively encoded by SACOL1493, is essential for the initiation step in DNA replication and is also involved in DNA repair (*33*). SACOL1707 codes for RadC, which plays an important role in DNA recombination of blocked or damaged replication forks (*34*). SACOL1955 encodes a DNA-damage-inducible protein P (DinP). At this point, it should be emphasized that Group I also had DNA repair-related genes, which exhibited expression level increases upon both 10 and 20 min exposures. Hence, this finding proposes that DNA repair mechanisms might be selectively induced to maintain DNA integrity for the synthesis of proteins vital for cell survival. Moreover, this versatile repair capability might be one of the schemes that allow *S. aureus* to resume growing even while part of the damage was apparently still being restored.

In Figure 4, we observed that the functional class of "amino acid biosynthesis" was also notable, which included SACOL2042-2043 (*ilvDB*), SACOL2045 (*ilvC*), and SACOL2046-2047 (*leuAB*) (Supporting Information Table 2). These genes are possibly part of an operon homologous to the *ilv-leu* operon encoding enzymes of branched-chain amino acid biosynthesis in *Bacillus subtilis* (*35*).

Group III: Genes Repressed upon 10 and 20 Min Exposures. Figure 3 shows that Group III consisted of 56 genes that were downregulated in response to both 10 and 20 min exposures. Of particular interest were the following genes: (i) SACOL0627 (*ung*) encodes uracil-DNA glycosylase (Ung) that initiates the repair of uracil DNA damage (*36*). (ii) SACOL1075-1076-1077 (*purCSQ*) is part of the *pur* operon, which encodes purine biosynthetic enzymes in *Bacillus subtilis* (*37*). (iii) SACOL0009 (*serS*), SACOL1148 (*pheS*), SACOL1710 (*valS*), SACOL1729 (*thrS*), and SACOL1778 (*tyrS*) are all related to aminoacyl tRNA synthesis. (iv) SACOL1328 (*glnR*)-SACOL1329 (*femC*) is associated with glutamine synthesis (*38*).

Group IV: Genes Repressed upon 10 Min Exposure. In Figure 3, 176 genes in Group IV exhibited mRNA level decreases at 10 min and no significant changes at 20 min. This group has the largest portion of the genes with statistically marked expression level changes. In Figure 4, one of the characteristics of Group IV was the presence of genes belonging to the functional classes of "cell envelope" and "transport and binding proteins". Supporting Information Table 2 shows that the genes of the "cell envelope" class are mostly involved in biosynthesis and degradation of surface polysaccharides and lipopolysaccharides. In addition, the genes of the "transport and binding proteins" class, including SACOL0620 (*proP*) and SACOL1384 (*opuD1*), primarily encode ABC transporter proteins that convey amino acids, peptides and amines.

Another noticeable result was that this group contained genes related to primary metabolism functions, including "amino acid biosynthesis", "biosynthesis of cofactors, prosthetic groups, and carriers", "central intermediary metabolism", "protein synthesis", and "purines, pyrimidines, nucleosides, and nucleotides" (Figure 4). For instance, SACOL0773 (*pabA*), SACOL1279 (*uppS*), SACOL1403-SACOL1404 (*trpEG*), SACOL1596 (*aroK*), and SACOL1977 (*pheA*) are involved in phenylalanine, tyrosine, and tryptophan biosynthesis, whereas SACOL1572 (*accB*) and SACOL2574 participate in pyruvate metabolism (Supporting Information Table 2). This finding might reflect general changes in cellular physiology and a metabolic repression as a result of exposure to peracetic acid. Furthermore, it can be speculated that the repression of these genes interfered with part of the respiratory metabolic pathways, which may be associated with the growth arrest upon 10 min exposure.

In Supporting Information Table 2, the functional class of "DNA metabolism" consisted of 8 genes in Group IV. Notable genes include SACOL0526, which putatively encodes DNA polymerase; SACOL0860 (*nuc*), which encodes a thermonuclease precursor; SACOL1150 (*rnhC*), which encodes a ribonuclease; and SACOL1542, SACOL1724, and SACOL2500 encode MutT/nudix family proteins, which play a role in cleansing the cell of potentially deleterious endogenous metabolites and modulating the accumulation of intermediates in biochemical pathways (*39*).

Supporting Information Table 2 also shows that several genes related to the pathogenesis of *S. aureus* were repressed after 10 min. These genes mainly belonged to the functional class of "cellular process". In detail, it was previously revealed that SACOL0743 (*bacA*) is required for virulence in *S. aureus* (*40*). The HtrA protein encoded by SACOL1028 is involved in controlling the expression of crucial secreted virulence factors that contribute to bacterial dissemination (*41*). SACOL1877 (*epiB*) participates in the biosynthesis of epidermin, which is a member of the lantibiotics, a group of peptide antibiotics (*42*). SACOL2350-2352 (*tcaBA*) plays an essential role in glycopeptide resistance in *S. aureus* (*43*). In addition, Aur encoded by SACOL2659 is a metalloproteinase, one of the major extracellular proteases, which are also important virulence factors in *S. aureus*. Last, SACOL2689-2690-2691-2692 (*icaADBC*) of the class of "cell envelope" mediates polysaccharide intercellular adhesion (PIA) production in *S. aureus*, which leads to cell−cell adhesion and is required for biofilm formation (*44*). This finding may propose the possibility that peracetic acid insult attenuated biofilm formation, which depends on the activity of the *icaADBC* locus.

Group V: Genes Induced upon 20 Min Exposure. In Figure 3, Group V had 123 genes that exhibited expression level increases only in response to 20 min exposure. One of the distinctions of Group V was the induction of genes related to the iron transport system in the functional classes of "cell envelope", "cellular processes", and "transport and binding proteins" (Figure 4). To be specific, SACOL0097 (*sirC*) and SACOL0099 (*sirA*) code for proteins involved in iron-siderophore (iron-chelating compound) import in *S. aureus* (*45*), whereas SACOL0105 codes for siderophore biosynthesis proteins. Siderophore-mediated iron uptake is one of the most important mechanisms that bacteria employ to acquire iron from the environment (*46*). Besides, SACOL2010 and SACOL2277 are iron compound transporters or iron compound-binding proteins. Notably, SACOL1141-1146 comprises the *isd* (*i*ron-responsive *s*urface *d*eterminant) operon, controlled by Fur (*f*erric *u*ptake *r*epressor), which is involved in bacterial heme iron uptake (*47*). Molecular iron is vital for aerobic respiration, DNA replication, and pathogenesis, and further, host proteins sequester iron and reduce the free iron concentrations within tissues to prevent bacterial growth. On the other hand, iron also promotes the formation of hydroxyl radicals, which indiscriminately damage all cellular components (*48*). In addition, superoxide, generated during the reduction process of oxygen, releases free iron from iron−sulfur proteins, thus increasing the levels of intracellular free iron (*49*). Therefore, iron metabolism is coordinately regulated with oxidative stress defenses. Our finding that the iron acquisition-related genes were more aggressively induced after 20 min may indicate that the iron uptake system was attenuated to prevent further oxidative damage caused by peracetic acid and/or was initially inactivated by increased intracellular iron concentration resulting from the oxidative damage, but the uptake resumed subsequently for the cellular growth after normal iron level was restored.

Figure 4 also shows the upregulation of genes belonging to the class of "DNA metabolism" after 20 min, which mainly participate in DNA replication and repair (Supporting Information Table 2). First, SACOL0780 (*recQ1*) encodes a protein belonging to the RecQ family of DNA helicases, which plays critical roles in maintaining genomic stability (*50*). SACOL1381 codes for SbcD, which participates in removing sequences that may interfere with DNA replication (*20*). SACOL1400 codes for an ImpB/MucB/SamB family protein, which is involved in UV protection. DNA primase DnaG, encoded by SACOL1619, initiates synthesis of the RNA primers required for DNA replication through a transient protein−protein interaction with the DNA helicase in *E. coli* (*51*). Last, SACOL1643 (*holA*) encodes the DNA polymerase III holoenzyme, essential for DNA replication (*52*). At present, it should be highlighted that DNA metabolism-related genes were dynamically regulated between the initial growth arrest and the subsequent partial recovery. That is, while DNA repair genes of Group I such as *uvrABC* were continuously induced at both growth states, the upregulation of some other repair genes including *recG* and *radC* was accompanied with the

growth arrest. Moreover, the abovementioned genes, which were associated with DNA replication, were upregulated along with the partial recovery. Therefore, it can be speculated that damaged DNA was restored enough for cells to synthesize DNA more actively at 20 min. For example, the fact that the *radC* gene, which encodes a protein that repairs damaged replication forks (*34*), showed induction at 10 min but no expression level change at 20 min might indicate that this lesion was already repaired upon 20 min exposure, which certainly allows cells more vigorous DNA replication and, consequently, the partial growth recovery.

Supporting Information Table 2 shows genes belonging to the class of "regulatory functions". To be specific, SACOL0179 encodes a phosphosugar-binding transcriptional regulator, while SACOL1611 codes for an iron uptake regulator. SACOL0725 and SACOL2378 encode arabinose operon regulators. Interestingly, SACOL2506 (*sarT*) encodes a repressor of alpha-hemolysin, an important extracellular virulence determinant of *S. aureus* (*53*). Last, Group V had SACOL2516 (*gntR*) coding for a transcriptional regulator, which represses gluconate uptake and initial catabolism in *E. coli* (*54*). Note that SACOL2514 (*gntP*) and SACOL2515 (*gntK*) were also found in Group V, which suggests that SACOL2514-2515-2516 may be organized in an operon.

In Supporting Information Table 2, we also observed that genes encoding major surface adhesion proteins associated with the pathogenesis of *S. aureus* were upregulated upon 20 min. In detail, SACOL2509 (*fnbB*) and SACOL2511 (*fnbA*) code for fibronectin-binding proteins, which mediate adhesion to fibronectin and invasion of mammalian cells, including epithelial, endothelial, and fibroblastic cells (*55*). SACOL2652 (*clfB*) encodes a clumping factor (fibrinogen-binding protein), a virulence factor in the pathogenesis of staphylococcal endocarditis (*18*). Further, SACOL1168 (*efb*) encodes a virulence factor that binds to both the complement C3b and fibrinogen, which inhibits complement activation and blocks opsonophagocytosis (*56*). Last, SACOL1169, adjacent to *efb*, also codes for a fibrinogen-binding protein precursor-related protein. This result suggests that surface adhesion activity, which enhances *S.* aureus virulence, be induced, while cells partially recovered from the growth arrest.

Group VI: Genes Repressed upon 20 Min Exposure. In Figure 3, Group VI comprised 71 genes with decreased mRNA levels only at 20 min. One characteristic of this group was the dominant presence of genes related to primary cellular metabolism. Especially, the functional classes of "energy metabolism", "fatty acid and phospholipid metabolism", and "purines, pyrimidines, nucleosides, and nucleotides" were distinctive (Figure 4). For example, SACOL1123 (*pyc*) is associated with the TCA cycle. SACOL0987 (*fabH*), SACOL0988 (*fabF*), SACOL1244 (*fabD*), and SACOL1245 (*fabG1*) encode proteins that play essential roles in Type II fatty acid biosynthesis, the principal route for the production of membrane phospholipid acyl chains in bacteria, which is regarded as a unique target for drug discovery (*57*). Besides, SACOL1210-1216 (*pyrR-uraA-pyrB-pyrC-carA-carB-pyrF*), which might be organized in an operon, is probably involved in the pyrimidine nucleotide biosynthesis (*58*). In relation to this finding, genes in the "protein synthesis" class, responsible for the synthesis of ribosomal proteins and aminoacyl tRNA, were repressed upon 20 min: SACOL0439 (*rpsR*), SACOL1001 (*trpS*), SACOL1137 (*rpmF*), SACOL1209, SACOL1238 (*rpmB*), and SACOL1642 (*rpsT*) (Supporting Information Table 2). Furthermore, it should be pointed out that the profiles of primary metabolism-related genes that were downregulated were considerably different between the two exposure times. Thus, we propose that those cellular metabolism genes repressed at 10 and 20 min may contribute to the initial growth arrest and the subsequent attenuated growth, respectively.

Supporting Information Table 2 shows that the functional class of "cellular processes" included SACOL1970 (*ssB2*), SACOL2025 (*argC2*), and SACOL2194 (*hysA*), which are related to the pathogenesis of *S. aureus*. The *ssB2* gene encodes a proteolytic enzyme that is important for infection in *S. aureus* (*59*); ArgC2 is a member of the accessory gene regulator locus that influences the expression of many virulence genes (*60*); and the *hysA* gene codes for a virulence factor which is essential in the early stages of subcutaneous infections (*61*).

Comparison with Transcriptome Response of *P. aeruginosa*. Last, to further understand bacterial response to peracetic acid, we compared the transcriptome profile of *S. aureus* in this paper with that of *P. aeruginosa*, a Gram-negative pathogen, (*7*) upon exposure to peracetic acid. Note that 1 mM peracetic acid was used in both of the studies. One of the most striking results that we revealed from this comparison analysis was that genes encoding oxidant defense enzymes such as glutathione peroxidase are highly upregulated in *P. aeruginosa* (*7*) but not in *S. aureus*. For instance, first, a glutathione peroxidase gene, PA2826, is upregulated by 16-fold in *P. aeruginosa*, whereas the corresponding genes of *S. aureus*, SACOL1325 (*gpxA1*) and SACOL2641 (*gpxA2*), showed no significant changes in mRNA levels. Second, organic resistance genes, SACOL0872 and SACOL1771, were not strongly induced in *S. aureus* (~2-fold); however, PA2850 (*ohr*) shows a 12-fold increase in the expression level in *P. aeruginosa*. Third, PA0848 (*ahpB*) and PA0140 (*ahpF*), which encode alkyl hydroperoxide reductases, are induced by 4- and 2-fold, respectively, in *P. aeruginosa*, while SACOL0451 (*ahpF*) was not among differently regulated genes in this study. However, housekeeping antioxidant genes such as *katA*, *ahpC*, and superoxide dismutase genes, which encode enzymes countering oxidant production during aerobic respiration, showed high transcription levels but no significant upregulation in both bacteria. Among these genes were PA4236 (*katA*), PA0139 (*ahpC*), PA4468 (*sodM*), and PA4366 (*sodB*) of *P. aeruginosa* and SACOL1368 (*katA*), SACOL0452 (*ahpC*), SACOL0118 (*sodA1*), and SACOL1610 (*sodA2*) of *S. aureus*.

Other intriguing results include the following: (i) tRNA-related genes were downregulated in both pathogens. As described above in Groups III and VI, genes encoding aminoacyl tRNA synthetase, such as *serS* and *trpS*, showed decreased transcription levels in *S. aureus*. On the other hand, peracetic acid represses the expression of tRNA accepting alanine, asparagine, histidine, isoleucineleucine, lysine, tyrosine, and valine in *P. aeruginosa* (*7*); (ii) DNA damage-related genes were commonly induced in *P. aeruginosa* and *S. aureus*. As detailed before, peracetic acid induced a number of such genes (e.g. *dinP*, *recG*, and *uvrAB*) in *S.* aureus. Likewise, PA0610-0611 (*prtNR*), PA0923 (*dinP*), and PA4763 (*recN*), which are all DNA damage-responsive genes, show increased expression levels in *P. aeruginosa* (*7*); and (iii) virulence factor genes were also induced in both bacteria even though the extent was much more significant in *S. aureus*. That is, genes encoding a wide range of virulence enzymes such as alpha toxin, exotoxins, and surface adhesion proteins were upregulated in *S. aureus* (see above), while exoenzyme genes exhibited an increase in expression levels in *P. aeruginosa*.

In summary, this paper described for the first time how *S. aureus* responded upon exposure to 1 mM peracetic acid with whole-genome microarrays. Furthermore, we reported how the transcriptome profile of *S. aureus* changed during its cellular response, which involved the growth arrest (10 min) and partial resumption (20 min). We also compared transcriptome responses to peracetic acid between *S.* aureus and *Pseudomonas aeruginosa*. Briefly, our microarray data

suggest the following conclusions. First, the regulation of membrane transport genes was significantly altered during the response. That is, ABC transporter and permease genes related to the passage of amino acids, carbohydrates, irons, and nucleosides were dynamically induced or repressed between the growth arrest and subsequent partial recovery. Second, genes involved in DNA repair and replication systems were selectively regulated between both growth states; certain repair genes (e.g. *uvrABC*) were continuously upregulated, and some others (e.g. *radC*) were induced only during the growth arrest, whereas the upregulation of DNA replication-related genes was primarily accompanied with the partial recovery. Third, different sets of primary metabolism-related genes were downregulated between the two growth states, implying that the respective genes might be associated with the initial growth arrest and the following attenuated growth. The last and perhaps most intriguing finding was that peracetic acid insult induced a wide range of virulence-related genes. These genes were differently regulated throughout the exposure times. For example, while alpha toxin and exotoxin genes were upregulated at both growth states, the induction of genes encoding major surface adhesion was accompanied with the growth recovery. However, interestingly, polysaccharide intercellular adhesion genes, associated with biofilm formation, were downregulated. This result suggests that the pathogenic schemes be selectively controlled in *S. aureus*, and many virulence factors be *indeed* produced upon exposure to peracetic acid, which was not extensively observed in *P. aeruginosa*. Hence, we are currently identifying signature genes that are commonly activated in other pathogenic bacteria to reveal general organizational and regulatory information regarding the disinfectant activity of peracetic acid. Finally, this information can be used to design new more effective methods of peracetic acid application for disinfecting pathogens. For instance, the result that *S. aureus* and *P. aeruginosa* showed dissimilarities in their transcriptome responses to peracetic acid may imply that optimal application schemes of peracetic acid might be different against different pathogenic bacteria.

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Supporting Information Available

Transcript level comparison of *Staphylococcus aureus* virulence-related genes between real-time PCR analysis and microarray analysis [The results are the mean of three biological replicates with three technical replicates each (± standard error) for each gene.] (Table 1) and *Staphylococcus aureus* genes showing statistically significant mRNA level changes upon either 10 or 20 min exposure to peracetic acid [The genes were grouped based on their regulation directions upon 10 and 20 min exposures.] (Table 2). This material is available free of charge via the Internet at http://pubs.acs.org.

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