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Toxicogenomic Response to Chlorination Includes Induction of Major Virulence Genes in *Staphylococcus aureus*

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Despite the widespread use of chlorination for microbial control in aqueous environments, cellular response mechanisms of human pathogens, such as *Staphylococcus aureus*, against chlorination remain unknown. In this work, genome-wide transcriptional analysis was performed to elucidate cellular response of *S. aureus* to hypochlorous acid, an active antimicrobial product of chlorination in aqueous solution. Our results suggest that hypochlorous acid repressed transcription of genes involved in cell wall synthesis, membrane transport, protein synthesis, and primary metabolism, while amino acid synthesis genes were induced. Furthermore, hypochlorous acid induced transcription of genes encoding major virulence factors of *S. aureus*, such as exotoxins, hemolysins, leukocidins, coagulases, and surface adhesion proteins, which all play essential roles in staphylococcal virulence. This work implies that chlorination may stimulate production of virulence factors, which provides new insight into host−pathogen interactions and effects of chlorine application for microbial control.

Introduction

Chlorination is the most widely used disinfecting process for drinking water, water distribution systems, swimming pools, wastewater processing, and food-processing environments (*1*, *2*). The majority of the disinfectants that are registered with U.S. Environmental Protection Agency (EPA) also exploit chlorination for their antimicrobial action. This widespread use of chlorination stems from the formation of hypochlorous acid (HOCl) in aqueous solution, a highly reactive oxidant that damages a variety of cellular materials (*3*). Interestingly, this antimicrobial activity of HOCl is also at the center of host−pathogen interaction. That is, when pathogens infect mammalian host cells such as human cells, they encounter the antimicrobial activity of HOCl, generated by the interaction of hydrogen peroxide with phagocyte-derived peroxidases (*4*). While host cells utilize HOCl as one of the defense mechanisms against infection, pathogens protect themselves from the lethality of HOCl by employing complex antioxidant strategies that serve to neutralize and repair oxidative damage. However, despite having been previously studied (*1*, *5*), the mode of action of HOCl and the corresponding response mechanisms of human pathogens are largely unknown (*6*, *7*).

To advance our understanding in this unknown territory, we investigated HOCl-driven changes in global genome expression in *Staphylococcus aureus* by using whole-genome microarrays and quantitative polymerase chain reaction (PCR). This genomic approach is particularly important given that pathogens can alleviate the lethal effects of HOCl despite a lack of specific enzymatic mechanisms for the detoxification of HOCl (*6*). Further, *S. aureus*, a Gram-positive bacterium, was selected as a model system because (i) *S. aureus* is a human pathogen responsible for a variety of diseases (*8*), ubiquitous in hospital facilities and aqueous environments (*9*), and (ii) the response of Gram-positive bacteria to HOCl is poorly understood (*6*). In this study, we show that all the major virulence genes of *S. aureus*, such as exotoxins, hemolysins, leukocidins, coagulases, and surface adhesion proteins, were induced upon exposure to HOCl. This result demonstrates that staphylococcal pathogenesis may be increased during host−pathogen interaction and during chlorine application for microbial control.

Materials and Methods

S. aureus cultures were initiated and maintained in Luria−Bertani (LB) broth at 37 °C as described in our previous papers (*10*, *11*). For growth inhibition, 0.0175% (2.4 mM) sodium hypochlorite (Aldrich Chemical Co., St. Louis, MO) was added to *S. aureus* cultures in a midexponential phase (OD_{600} = 0.8). OD_{600} was measured by using Lambda 25 spectrophotometer (Perkin-Elmer, Inc., MA). In addition, cell viability was assessed by enumerating viable colonies on LB-agar plates. Note that the pH of *S. aureus* cultures was around 7.0 at 37 °C after the exposure, which indicates HOCl predominates over hypochlorite ion (OCl^-) in the cultures (*12*). However, to avoid confusion, we use the term HOCl to include both HOCl and hypochlorite ion in this paper.

Total RNA was isolated after 10 and 20 min incubation using the RiboPure − Bacteria kit (Ambion, Inc., Austin, TX) (*13*). RNA quality was determined using both Lambda 25 spectrophotometer (Perkin-Elmer, Inc.) and RNA 6000 Nano LabChip with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). cDNA synthesis, cDNA fragmentation, labeling, hybridization, staining, and washing steps were performed according to the manufacturer's protocol for the Affymetrix *S. aureus* GeneChip arrays (Affymetrix, Inc., Santa Clara, CA). The arrays were scanned with the Affymetrix GeneChip Scanner 3000. To analyze the array data, GeneChip Operating Software (GCOS) v. 1.2 (Affymetrix, Inc.) and GeneSpring GX v. 7.3 (Agilent Technologies, Inc.) were utilized with the following parameters: *alpha* 1, 0.04; *alpha* 2, 0.06; *tau*, 0.015; target signal, 500. Fold changes were calculated as the ratio between the signal averages of three biological controls (untreated) and three biological experimentals (HOCl-treated) for 10 and 20 min exposures.

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

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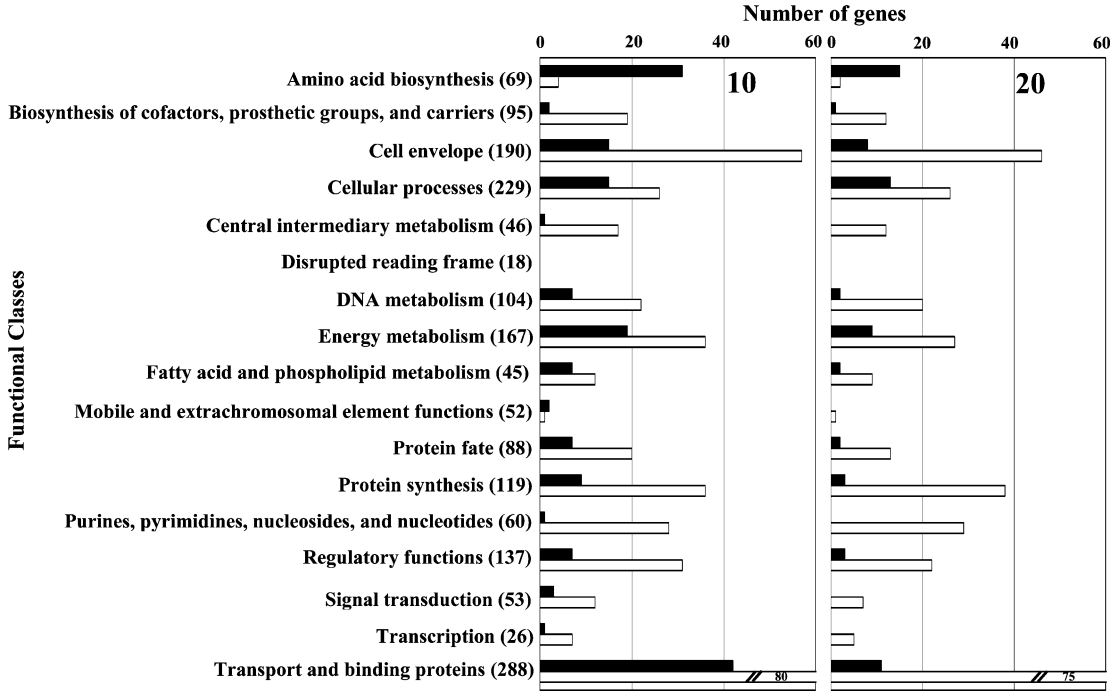

FIGURE 1. Functional classification of genes with statistically significant increase (■) and decrease (□) in mRNA level upon 10 and 20 min exposures (a total of 1086 genes). The number in parenthesis represents the total number of genes within the genome in each functional class.

control (*14*). The real-time PCR was performed by employing iCycler iQ Real-Time PCR Detection System with iScript cDNA Synthesis Kit and 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 95.0 °C, followed by 40 cycles of 10 s at 95.0 °C, 30 s at 55.0 °C, and 20 s at 72.0 °C. PCR efficiencies were derived from standard curve slopes in the iCycler software v. 3.1 (BioRad Laboratories, Inc.). 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

To study the effect of HOCl-induced stress on *S. aureus*, we performed a transcriptome analysis upon exposure to sodium hypochlorite by using whole-genome microarrays. *S. aureus* was exposed to 2.4 mM sodium hypochlorite because we confirmed that this concentration caused sublethal inhibition on cell growth for the first 20 min posttreatment (data not shown). This concentration was selected also based on the fact that U.S. Government regulations recommend a maximum concentration of 2.7 mM of sodium hypochlorite for disinfecting food processing equipment and food contact surfaces (*15*), and a similar level of concentration of sodium hypochlorite (7.2 mM) is recommended for eradicating bacterial pathogens and virus in blood samples, which are commonly present in hospital environments (*16*). Note that the minimum inhibitory concentration (MIC) of sodium hypochlorite is 2.0 mM for *S. aureus* (*17*). 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 GEO Series accession number GSE5498.

To 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 datasets. First, we selected probe sets that had a presence or marginal detection call (*18*) from 50% or more biological replicates on both the experimental and control replicate sets. McClintick and Edenberg recently reported that this filtering effectively eliminates false positive probe sets in the Affymetrix GeneChip system (*19*). Second, we then applied a *t*-test to these probe sets with a cutoff *p*-value of 0.05. Finally, we selected open reading frame (ORF) probe sets with a two or higher fold change in transcript level. As a result, 287 and 685 genes showed statistically marked increases and decreases in mRNA levels, respectively, after 10 min treatment. Upon 20 min exposure, 124 and 560 genes exhibited statistically marked expression level increases and decreases, respectively. Note that among these genes, 570 genes showed statistically marked changes upon both 10 and 20 min exposures. Thus, a total of 1086 genes were differently expressed in response to either 10 min or 20 min exposure.

To examine how genes with transcript level changes are distributed with regard to their functions, we further classified these 1086 genes according to the categories described in the comprehensive microbial resource (CMR) of the Institute of Genome Research (TIGR) (http://www.tigr.org/tigr-scripts/CMR2/CMRHomePage.spl/). Note that we also referred to the operon prediction information of TIGR for an operon analysis throughout the discussion. Figure 1 shows the number of differentially regulated genes in each functional class, and the total number of genes within the genome in each functional class because each functional class constitutes a different fraction of the genome. Finally, to identify genes with similar transcription patterns during the time course, we classified the differentially regulated 1086 genes into six groups on the basis of their transcription directions. Figure 2 displays the number of genes of Groups I through VI in each functional class. In addition, Supporting Information Table 2 displays the genes of each group, and their fold changes and *p*-values in response to 10 and 20 min exposures.

Group I: Genes Induced upon 10 and 20 Min Exposures. Supporting Information Table 2 shows that 109 genes in Group I were upregulated upon both 10 and 20 min

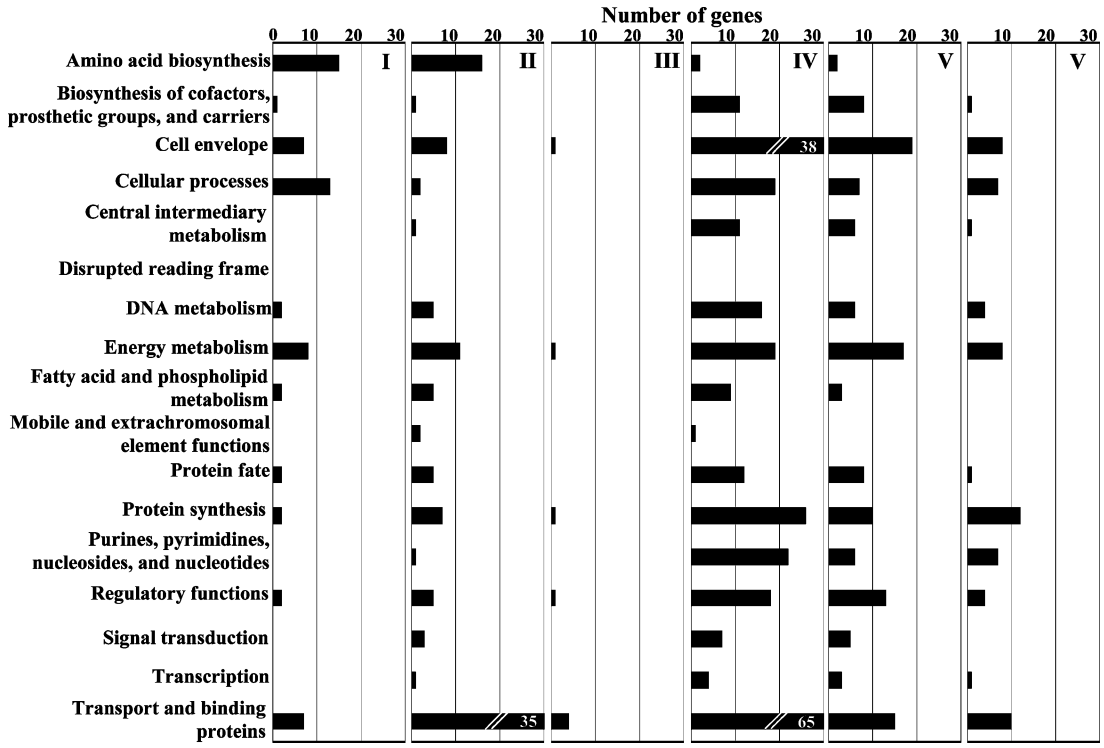

FIGURE 2. The number of genes within Groups I through VI in each functional class. Note that the functional classes of "hypothetical proteins" and "unknown function" are not included in this figure.

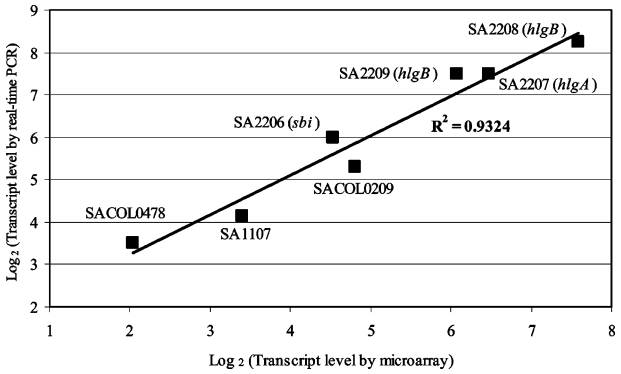

FIGURE 3. Transcript level comparison of virulence factor genes of *S. aureus* between real-time PCR and microarray analyses. The results are the mean of three biological replicates with three technical replicates each for each gene.

exposures. The most striking result was the dominant presence of genes linked to the pathogenesis of *S. aureus*, primarily in the classes of "cellular processes" and "cell envelope" (Figure 2). These genes code for exotoxins, hemolysins, leukocidins, coagulases, and surface adhesion proteins, which all play essential roles in staphylococcal virulence (*20*). Note that the transcription level changes of these virulence genes were validated by quantitative real-time PCR analysis. Figure 3 shows that our microarray results were corroborated with real-time PCR analysis, which provides independent verification of transcript level changes of the genes in this study. First, SA0383 (*set7*), SA0389 (*set13*), SA1010, and SACOL0478 encode exotoxins responsible for food poisoning and toxic shock syndrome (*21*). Second, SA1007 encodes an alpha-hemolysin, which causes membrane damage to many types of mammalian cells (*20*), whereas a toxin encoded by SA1752 (*hlb*) is a beta-hemolysin, responsible for tissue necrosis (*22*). Further, SA2207-SA2208-SA2209 (*hlgACB*) encodes gamma-hemolysins that affect neutrophils and macrophages and lyse mammalian erythrocytes (*20*). Notably, *hlgACB* was among the genes that showed the highest expression increases in our study. Third, SA1812-SA1813 (*lukFM*) codes for leukocidin toxins that damage neutrophils and macrophages (*20*). Fourth, SACOL0209 encodes a coagulase, which is associated with local and hematogenous staphylococcal infections (*23*). Fifth, as for surface adhesion genes, SA2290 (*fnbB*)-SA2291 (*fnb*) encodes fibronectin-binding proteins, which mediate adhesion to fibronectin and invasion of mammalian cells, including epithelial, endothelial, and fibroblastic cells (*24*). SA1003-SA1004 putatively encodes clumping factors (fibrinogen-binding proteins), virulence factors in the pathogenesis of staphylococcal endocarditis (*25*). In addition, SA2206 (*sbi*) encodes an immunoglobulin-binding protein, which can mask the bacteria from the immune system of the host (*26*). Intriguingly, virulence-related enzymes are involved in microbial defense systems against oxidants by damaging phagocytes and/or impairing oxidants (*4*). Further, some of the virulence factors scavenge reactive oxygen species (*27*, *28*) Consistent with this notion, we previously demonstrated that peracetic acid, another oxidative disinfectant, induces virulence factor genes in *S. aureus* (*13*). These prior results in conjunction with our finding that HOCl induced major virulence factor genes imply that (i) among *S. aureus* virulence factors, some may function to defend against HOCl, and linked induction of these factors with other virulence determinants may result in the broad pattern of induction observed, and/or (ii) *S. aureus* may adopt the presence of HOCl as an environmental signal that triggers the expression of virulence determinants.

Group II: Genes Induced upon 10 Min Exposure. A quantity of 178 genes in Group II showed increased expression levels at 10 min; however, upon 20 min exposure, the expression level changes of these genes became statistically insignificant. Figure 2 indicates that one of the characteristics of Group II was the upregulation of 35 genes in the functional class of "transport and binding proteins". These genes encode

ATP-binding cassette (ABC) proteins that transport amino acids, or cations and iron-carrying compounds (Supporting Information Table 2). Interestingly, 20 of the 35 genes code for proteins associated with amino acids transport. In relation to this outcome, Figure 2 shows that 16 genes in the functional class of "amino acid biosynthesis" were upregulated at 10 min. Group I, the genes of which exhibited upregulation at both 10 and 20 min, also possessed 15 genes in the "amino acid biosynthesis" class. Therefore, this outcome in conjunction with the extensive regulation of the genes encoding amino acid transporters suggests the possibility that HOCl exposure might stimulate amino acid metabolism systems in *S. aureus*.

Supporting Information Table 2 shows that genes related to the pathogenesis of *S. aureus* were also induced at 10 min, which mainly belonged to the classes of "cellular processes" and "protein synthesis". To be specific, SA0116 and SA0117, organized in CMR's predicted operon, putatively encode proteins involved in the biosynthesis of the siderophore aerobactin, an important virulence component that sequesters iron from the host (*29*). Moreover, SA0388 (*set12*), SA0390 (*set14*), and SA1009 code for exotoxins, which are a major cause of staphylococcal disease, responsible for food poisoning and toxic shock syndrome (*21*). Exotoxins are excreted and remain extracellular even when the cells are killed, which contributes to staphylococcal food poisoning. This result, along with induction of all the major virulence genes of *S. aureus* in Group I, suggests that HOCl exposure may increase staphylococcal pathogenesis.

Supporting Information Table 2 also shows that Group II had DNA damage-related genes in the functional class of "DNA metabolism". In detail, SA0991 (*mutS2*) encodes a major component of the methyl-mismatch repair system (*30*). SA0993 (*uvrC*) codes for a protein that mediates nucleotide excision repair (*31*). Further, SA1286 putatively encodes the DnaD protein, which is essential for the initiation step in DNA replication and is also involved in DNA repair (*32*). SA1328 (*xerD*) codes for a site-specific recombinase that converts chromosome mutimers formed by homologous recombination back to monomers for cell division (*33*). Last, SA1711 putatively codes for a DNA polymerase involved in DNA repair. However, despite the upregulation of these genes as well as SA1285 (*nth*) of Group I, which is related to the base excision repair of DNA damage in *E. coli* (*34*, *35*), parts of DNA repair genes, especially in Group IV, were downregulated upon exposure to HOCl (discussed below).

Group IV: Genes Repressed upon 10 and 20 Min Exposures. Group IV was comprised of 461 genes downregulated in response to both 10 and 20 min exposures. This group represents the largest portion of the statistically marked 1086 genes in our study. Among the functional classes of Group IV, the most dominant class was "transport and binding proteins", which contained 65 genes (Figure 2). These genes primarily encode proteins that transport amino acids, peptides, and amines (26 genes), cations and iron-carrying compounds (10 genes), and carbohydrates (5 genes). Figure 1 shows that 80 and 75 genes in the functional class of "transport and binding proteins" were downregulated upon 10 and 20 min exposures, respectively. This result implies that active and/or facilitated transport through the cell membrane was significantly repressed upon exposure to HOCl in *S. aureus*.

Figure 2 shows that the functional class of "cell envelope" was also distinctive, which contained 38 genes. In particular, we observed the downregulation of SA2458 to SA2462 (*icaRADBC*), which consist of the intercellular adhesion (*ica*) operon and its transcriptional regulator gene (*icaR*). The *ica* operon mediates polysaccharide intercellular adhesion (PIA) production in *S. aureus*, which leads to cell−cell adhesion and is required for biofilm formation (*36*). Therefore, this finding proposes the possibility that HOCl insult attenuated biofilm formation, which depends on the activity of the *icaADBC* locus.

Of another importance was that Group IV had genes related to DNA repair, which belonged to the functional class of "DNA metabolism" (Supporting Information Table 2 and Figure 2). These genes included SA0538 (*ung*), SA1282 (*recU*), SA1853, and SA1350 (*recN*). Note that SA1350-SA1351-SA1352 (*recN-ahrC-ispA*) of Group IV is organized in CMR's predicted operon. Given that HOCl causes DNA damage (*3*−*5*), the result here that parts of the DNA repair genes of *S. aureus* were repressed is surprising. However, as observed in hydrogen peroxide-induced response in *S. aureus* (*10*), this finding may also suggest that the DNA repair system of *S. aureus* is selectively regulated upon exposure to HOCl. Alternatively, this result may be explained by the fact that the mechanism of action of HOCl is dose-dependent, where DNA damage primarily occurs at lower concentrations of HOCl (*10*).

Figure 2 shows that the functional class of "protein synthesis" was composed of 26 genes in Group IV, which are responsible for the synthesis of ribosomal proteins and aminoacyl-tRNA. Figure 1 indicates that this functional class has 119 genes in *S. aureus*; thus, about 22% of the genes of the "protein synthesis" class were downregulated at 10 and 20 min in response to HOCl. Aminoacyl-tRNA and ribosomal proteins are required for protein translation. Therefore, this result might reflect the overall reduction of protein synthesis and further, cellular metabolism during HOCl-caused growth inhibition.

In relation to metabolic repression, we also observed that 72 genes of Group IV were involved in primary metabolism functions (Figure 2). These genes were distributed over the functional classes of "biosynthesis of cofactors, prosthetic groups, and carriers", "central intermediary metabolism", "energy metabolism", "fatty acid and phospholipid metabolism", and "purines, pyrimidines, nucleosides, and nucleotides". Evidently, this result suggests that HOCl exposure led to the repression of genes participating in primary metabolic pathways, which might contribute to metabolic depression and further, growth inhibition at 10 and 20 min. Alternatively, this phenomenon can probably be described as one of enzyme regulatory activities. That is, since many metabolic enzymes are regulated at the transcriptional level by the end products of their respective metabolic pathways, it is also possible that increased level of metabolic end products contributed to the repression of these genes.

Group V: Genes Repressed upon 10 Min Exposure. A quantity of 224 genes in Group V exhibited mRNA level decreases at 10 min and no significant changes at 20 min. One of the characteristics of Group V was the repression of 19 genes belonging to the functional class of "cell envelope". In particular, genes related to cell wall synthesis were distinctive; thus, this result as well as the repression of cell wall synthesis genes in Group IV implies that HOCl might suppress the synthesis of cell wall-related systems in *S. aureus*.

Another notable finding of Group V was that 40 genes were involved in primary metabolism functions (Figure 2). As described above, these genes belonged to the functional classes of "biosynthesis of cofactors, prosthetic groups, and carriers", "central intermediary metabolism", "energy metabolism", "fatty acid and phospholipid metabolism", and "purines, pyrimidines, nucleosides, and nucleotides".

Group VI: Genes Repressed upon 20 Min Exposure. In Supporting Information Table 2, 99 genes in Group VI showed decreased mRNA levels only at 20 min. As shown in Figure 2, the most dominant class was "protein synthesis". Despite the overall repression of protein synthesis genes in response to HOCl (Figure 2), most of the protein synthesis genes of Group VI were responsible for ribosomal protein production,

such as SA0354 (*rpsR*), SA1067 (*rpmB*), and SA1099 (*rpsB*). In Figure 2, we also observed genes related to primary metabolism that mainly belonged to the functional classes of "energy metabolism", and "purines, pyrimidines, nucleosides, and nucleotides".

In summary, this paper describes the first genome-wide transcriptional analysis of *S. aureus* response to chlorination. The significance of the results presented in this paper lies in the fact that chlorination is the most widely used disinfecting process for microbial control and is also involved in host−pathogen interaction; however, the corresponding response mechanisms of microbes are unknown. Briefly, our data based on the toxicogenomic analysis showed the following results. First, the membrane function-related genes of *S. aureus* were repressed, implying that chlorination-induced damage might reduce active and/or facilitated transport through the cell membrane. Second, chlorination resulted in the repression of primary metabolism-related genes and protein synthesis genes, which might contribute to the growth inhibition. Alternatively, this phenomenon can be described as an enzyme regulatory activity, where increased levels of metabolic end products contributed to the repression of these genes. Third, chlorination induced one-third of the amino acid synthesis genes of *S. aureus*. This outcome implies that *S. aureus* may increase amino acid production upon exposure to chlorination. Most strikingly, we provided evidence that chlorination resulted in induction of the major virulence factor genes of *S. aureus*. These genes code for exotoxins, hemolysins, leukocidins, coagulases, and surface adhesion proteins, which all play essential roles in staphylococcal virulence. One important implication of our result is that *S. aureus* may generate virulence factors under phagocyte-driven HOCl stress during active infection, which increases staphylococcal pathogenesis and damages mammalian host cells. In this light, it is notable that inappropriate use of antibiotics contributes to worse antimicrobial outcomes by inducing and enhancing toxin production (*37*). Consequently, we propose that staphylococcal pathogenesis can be increased during phagocyte-driven chlorine stress and during chlorine application for microbial control.

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Supporting Information Available

(1) Genes and primer sequences employed for the real-time PCR analysis, and (2) S. aureus genes that showed statistically significant mRNA level changes upon either 10 or 20 min exposure to hypochlorite. This material is available free of charge via the Internet at http://pubs.acs.org.

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