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Global Transcriptome Analysis of *Staphylococcus aureus* Response to Hydrogen Peroxide†

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Staphylococcus aureus **responds with protective strategies against phagocyte-derived reactive oxidants to infect humans. Herein, we report the transcriptome analysis of the cellular response of *S. aureus* to hydrogen peroxide-induced oxidative stress. The data indicate that the oxidative response includes the induction of genes involved in virulence, DNA repair, and notably, anaerobic metabolism.**

Staphylococcus aureus is a gram-positive pathogen capable of causing a variety of diseases, ranging from benign skin infections to life-threatening endocarditis and toxic shock syndrome (53). The main habitats of this bacterium are the nasal membrane and skin of warm-blooded animals including humans, which also act as a primary line of protection against infection. However, when this pathogen enters the underlying tissues, innate host defense primarily mediated by macrophages plays a pivotal role (37). In particular, during active infection, macrophages and other lymphocytes use toxic reactive oxygen species such as hydrogen peroxide, superoxide, and hydroxyl radicals to destroy the phagocytosed bacteria.

Reactive oxidants cause damage to the essential biomaterials of cells; for instance, by reacting with intracellular iron, hydrogen peroxide can form the hydroxyl radical through the Fenton reaction, which injures various cellular molecules including lipids, proteins, and DNA (40). Superoxide is also capable of promoting oxidative damage by increasing the intracellular concentration of free iron (28). Even normal cellular metabolism produces cytotoxicity arising from its partially reduced intermediates (37). Therefore, in most of these environments, the resistance against reactive oxygen species is crucial for bacterial survival.

In order to cope with reactive oxidants, *S. aureus* is known to be equipped with a multifaceted defense system that includes such enzymes as catalase and superoxide dismutase (37). There are many specific defense genes that have been identified, and regulatory aspects of their activities have been revealed in many cases (58). However, in spite of this marked progress, a lack of understanding of the linkage between the cell's defense mechanism against reactive oxygen species and the remainder of the cell's metabolism hinders further development of more innovative methods for combating this pathogen. Better elucidation of the molecular events responsible for establishing and maintaining pathogenicity might improve drug and vaccine optimization (55). Consequently, there has been a necessity to provide a more complete linkage between cell physiology and the well-characterized defense response in *S. aureus*.

Microarray-based transcriptome analysis, which enables us to simultaneously and globally examine the complete transcriptional response at the genomic level, has been successfully used to explore the oxidative stress responses of *Pseudomonas aeruginosa* and *Escherichia coli* (9, 46, 63). In the present study, we used Affymetrix *S. aureus* GeneChip arrays to investigate the dynamics of global gene expression profiles during the cellular response of *S. aureus* to oxidative stress induced by hydrogen peroxide (10 mM), which involved initial growth inhibition (10 min) and subsequent recovery (20 min). To our knowledge, this is the first study demonstrating the transcriptome analysis of *S. aureus* response to oxidative stress, as well as the first for exposure to hydrogen peroxide. Consequently, the results presented herein may facilitate the further elucidation of the mechanisms involved in *S. aureus*-host interactions.

Affymetrix *S. aureus* GeneChip arrays. In this study, we used *S. aureus* NCTC 8325 obtained from the Network on Antimicrobial Resistance in *S. aureus*. To maintain homogeneous culture samples throughout our experiments, we used the following three steps (9, 10). (i) We initiated *S. aureus* cultures at 37°C with shaking at 250 rpm in sterilized Luria-Bertani (LB) broth (10 g of tryptone, 5 g of yeast extract, and 10 g of sodium chloride per liter). (ii) After 17 h, we diluted the overnight cultures 1:100 in prewarmed LB broth and incubated it at 37°C with shaking at 250 rpm until the optical density at 600 nm reached the early logarithmic phase (~0.8). (iii) We rediluted the cells 1:10 in prewarmed LB broth and incubated them at 37°C with shaking at 250 rpm. We added 10 mM hydrogen peroxide (Aldrich Chemical Co., St. Louis, MO) immediately after the optical density at 600 nm reached 0.8. Note that culture volumes for all growth conditions were adjusted to less than 1/10 of the total flask volume to maximize aeration. After 10 and 20 min of incubation, we isolated total RNA with the RiboPure Bacteria kit (Ambion, Inc., Austin, TX) by following the manufacturer's protocol. 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). To ana-

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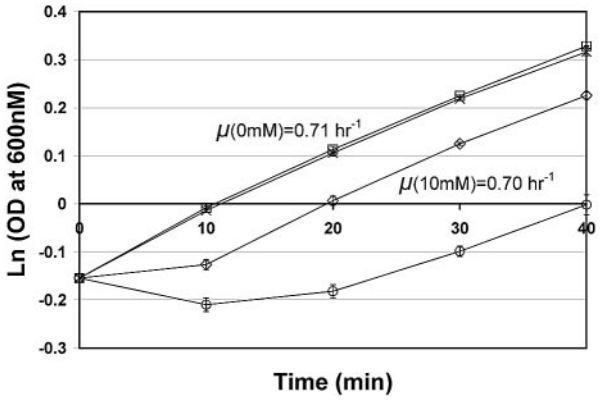

FIG. 1. *S. aureus* growth (optical density [OD] at 600 nm) after treatment with hydrogen peroxide at 0 mM (×), 1 mM (□), 10 mM (◇), or 40 mM (○). The cell growth rate (μ) with 0 mM or 10 mM hydrogen peroxide was calculated during the exponential phase. The results are the means of triplicate experiments; the error bars represent standard deviations.

lyze the array data, we used 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.

Validation of array data by real-time PCR. 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. For a list of the genes and primer sequences used for the real-time PCR analysis, see Table 2. The housekeeping gene 16S rRNA was used as an endogenous control (60). We performed the real-time PCR by using the iCycler iQ Real-Time PCR Detection System with an iScript cDNA Synthesis Kit and IQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA). For each gene, three biological replicates with three technical replicates each were used. Reaction mixtures were initially incubated for 3 min at 95°C, followed by 40 cycles of 10 s at 95°C, 30 s at 58.9°C, and 20 s at 72°C. PCR efficiencies were derived from standard curve slopes in the iCycler software v. 3.1 (Bio-Rad Laboratories, Inc., Hercules, CA). Melting 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 a 16S rRNA gene was used to determine transcript level changes.

Transcriptome changes in response to oxidative stress. To investigate the effect of sublethal oxidative stress on *S. aureus*, we performed a transcriptome analysis with microarrays upon exposure to hydrogen peroxide. Exponentially growing cells of *S. aureus* were exposed to various concentrations of hydrogen peroxide. Figure 1 shows that a 10 mM hydrogen peroxide insult triggered growth inhibition for about 10 min. After this inhibition time, cells continued to grow at the same rate as untreated cells. Therefore, in the present study, to better understand how *S. aureus* initially responds to oxidative stress and subsequently recuperates from the damage, we used 10- and 20-min exposures to 10 mM hydrogen peroxide.

To determine genome-wide transcriptional changes in response to hydrogen peroxide, we conducted three independent microarray experiments in the absence (control) or presence (experimental) of 10 mM hydrogen peroxide upon 10- and 20-min exposures. Moreover, to study the effects of hydrogen peroxide treatment on *S. aureus*, we used separate sets of untreated controls for 10 and 20 min. Another reason for such an experimental design was that we had found a difference in the transcriptional profiles of untreated controls between the two exposure times.

To further identify genes with statistically significant changes in expression levels, we applied the following criteria to each of the 10-min and 20-min control experimental microarray data sets: (i) a *P* value for a *t* test should be less than 0.05, (ii) an absolute change in transcript level should be equal to or greater than twofold, and (iii) a gene should have a presence or marginal call from 50% or more of the replicates in both the experimental and control replicate sets for each of the 10- and 20-min conditions. As a result, we found that 113 and 151 genes showed statistically significant increases and decreases in mRNA levels, respectively, after 10 min of treatment. Upon 20 min of exposure, 95 and 24 genes exhibited statistically significant expression level increases and decreases, respectively. Note that among these genes, 40 genes showed statistically significant changes upon both 10- and 20-min exposures. Therefore, 343 genes were differently expressed in response to either 10 min or 20 min of exposure.

To examine how genes with transcript level changes are distributed with regard to their functions, we further classified these 343 genes according to the categories described in the comprehensive microbial resource of The Institute of Genome Research (http://www.tigr.org/tigr-scripts/CMR2/CMRHomePage.spl/). Figure 2 shows the number of differentially regulated genes in each functional class. The most noticeable feature was that the total number of downregulated genes dramatically decreased upon 20 min of exposure. On the other hand, the numbers of upregulated genes were similar between the 10- and 20-min exposures, although the dominant functional classes were different. This result demonstrates that the transcriptional responses of *S. aureus* are significantly different between the 10- and 20-min exposures to 10 mM hydrogen peroxide, and in particular, considerably fewer genes were repressed upon 20 min of exposure. Furthermore, this shift in the transcriptional profiles may account for the initial growth inhibition and the growth recovery.

Classification of genes by their regulation patterns. To identify genes with similar transcription patterns during the time course, we classified the 343 differentially regulated genes into seven groups on the basis of their transcription directions. Table 1 shows the genes in each group and their *n*-fold changes and *P* values in response to 10- and 20-min exposures. Note that genes belonging to the functional classes of "hypothetical proteins," "hypothetical proteins-conserved," and "unknown function" are not included in Table 1. The complete description of all 343 genes is displayed in Table S1 in the supplemental material. Briefly, group I contained 20 genes upregulated upon both exposure times, while group II had 92 genes with increased expression levels at 10 min and no significant changes upon 20 min of exposure. Further, group III possessed seven genes that were induced and repressed in response to 10- and 20-min exposures, respectively. Group IV contained 132 genes downregulated after 10 min, whereas 12 genes of group

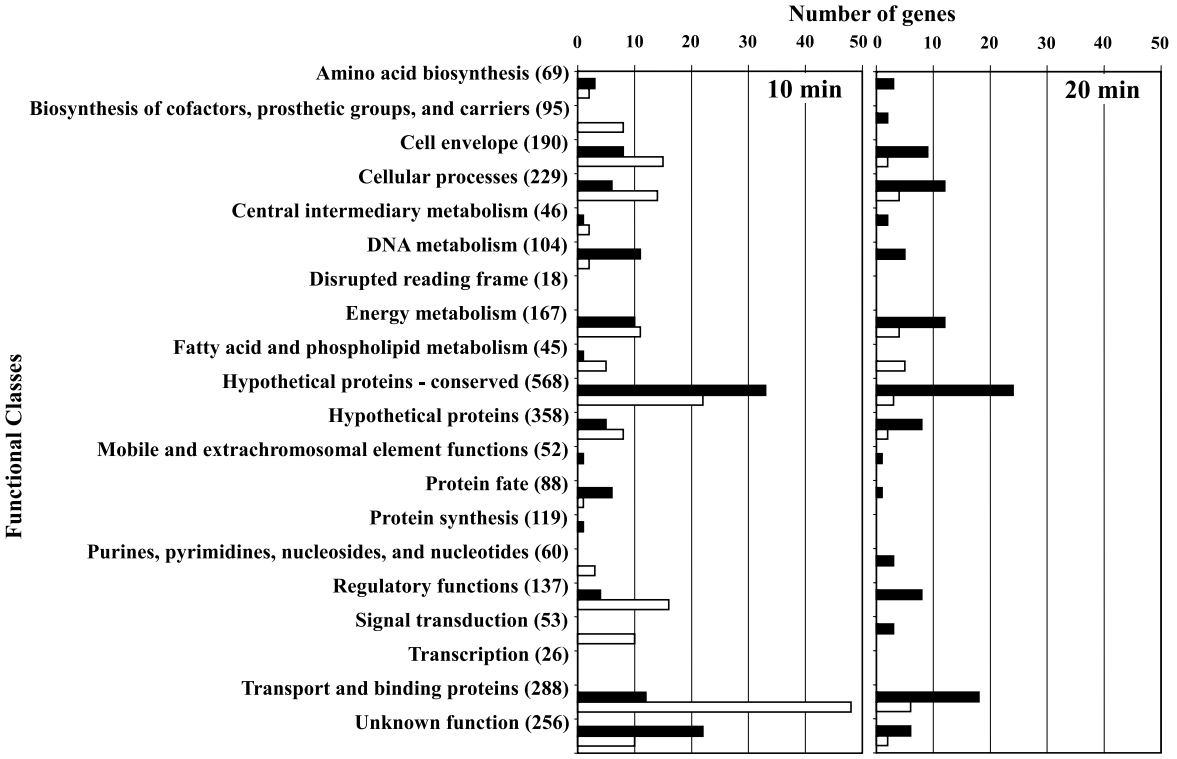

FIG. 2. Functional classification of genes with statistically significant increases (■) and decreases (□) in mRNA level upon 10-min and 20-min exposures to hydrogen peroxide (a total of 343 genes). The number in parenthesis represents the total number of genes within the genome in each functional class.

V exhibited decreased mRNA levels upon both exposure times. Finally, groups VI and VII had 68 and 12 genes that were induced and repressed, respectively, upon 20 min of exposure.

Group I was composed of 20 genes that were induced upon both 10- and 20-min exposures. One of the characteristics of this group was the induction of DNA repair-related genes COL-SA0823 (*uvrB*), -SA0824 (*uvrA*), -SA1374 (*lexA*), -SA1400, and -SA2131 (Table 1). Specifically, *uvrA* and *uvrB* encode proteins required during the early steps of nucleotide excision repair to form a DNA-protein complex at the damaged site that allows incision to occur (24). The *lexA* gene encodes a repressor protein regulating the SOS response genes, which include DNA repair and recombination genes (2, 3). In relation to this gene, we also observed that COL-SA1304, which codes for RecA, was upregulated upon 20 min of exposure (group VI). This gene was also induced by 2.3-fold in response to 10 min of exposure, but the *P* value (0.079) was higher than the cutoff. The RecA protein stimulates the autocatalytic cleavage of LexA and thus increases the expression of the genes of the SOS regulon (3). Congruent with this finding, increased expression of *recA* and *lexA* because of hydrogen peroxide treatment was previously reported in *E. coli* and *P. aeruginosa* (46, 63). Besides, COL-SA1400 codes for an ImpB/MucB/SamB family protein which is involved in UV protection, whereas 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 (61). Consequently, our data corroborate previous studies that have associated oxidative stress response genes with hydrogen peroxide and other reactive oxidants and reinforce the conclusion that DNA repair proteins may be among the most central mechanisms that *S. aureus* uses to counteract lethal effects of reactive oxygen intermediates. More importantly, our data suggest that the DNA repair system was continuously activated even after the growth of *S. aureus*, which had been initially inhibited by hydrogen peroxide, resumed at the same rate as that of untreated cells.

Group II consisted of 92 genes that were upregulated at 10 min; however, upon 20 min of exposure, the expression level changes of these genes became statistically insignificant. As shown in Table 1, this group also had a number of genes belonging to the functional class of "DNA metabolism." Specifically, COL-SA1241 (*recG*) is involved in the repair of DNA damage resulting from quinolone treatment in *S. aureus* (43). The Nth protein (endonuclease III) encoded by COL-SA1492 is a DNA glycosylase involved in the first step of base excision repair of DNA damage in *E. coli* (16, 31). Moreover, the DnaD protein, putatively encoded by COL-SA1493, is essential for the initiation step in DNA replication and is also involved in DNA repair (34). As discussed above, group I also had DNA repair-related genes which exhibited expression level increases upon both 10- and 20-min exposures, whereas the genes here showed increases only at 10 min of exposure. Therefore, this result suggests that DNA repair mechanisms are selectively induced to maintain DNA integrity for the synthesis of proteins vital for cell survival. For example, since the *nth* gene is related to oxidative pyrimidine damage (31), no significant

TABLE 1. *S. aureus* genes that showed statistically significant mRNA level changes upon either 10 or 20 min of exposure to hydrogen peroxide[a]

Group and ORF[b]	Gene	10 min		20 min		Functional class
		n-Fold change	*P* value	*n*-Fold change	*P* value	
I. Upregulation (10 min)-upregulation (20 min)						
COL-SA0244	*scdA*	4.1	0.032	10.1	0.014	Cellular processes
COL-SA2131		7.5	0	13.8	0.015	Cellular processes
COL-SA0823	*uvrB*	4.3	0.01	3.9	0.015	DNA metabolism
COL-SA0824		2.6	0.003	3.5	0.024	DNA metabolism
COL-SA1400	*uvrA*	8.5	0.018	17.7	0.036	DNA metabolism
COL-SA1374	*lexA*	4.4	0.009	4.5	0	DNA metabolism; regulatory functions
COL-SA0494	*nuoF*	2.0	0.009	2.1	0.029	Energy metabolism
COL-SA0321		2.3	0.042	2.1	0.043	Mobile and extrachromosomal element functions
COL-SA2563		5.6	0	7.9	0.02	Protein fate
II. Upregulation (10 min)-no change (20 min)						
COL-SA0502		13.4	0.01			Amino acid biosynthesis
COL-SA0503		10.6	0.03			Amino acid biosynthesis
COL-SA0557	*cysK*	2.5	0.009			Amino acid biosynthesis
COL-SA0138	*cap5C*	2.0	0.012			Cell envelope
COL-SA0466		2.5	0.004			Cell envelope
COL-SA1161	*murI*	2.0	0			Cell envelope
COL-SA1522		2.6	0.033			Cell envelope
COL-SA2002	*map*	2.1	0.035			Cell envelope
COL-SA2554		2.9	0.022			Cell envelope
COL-SA2412		2.6	0.026			Cell envelope; transport and binding proteins
COL-SA1179		2.1	0.003			Cellular processes
COL-SA1180		3.1	0.008			Cellular processes
COL-SA1003		2.0	0.036			Cellular processes; regulatory functions
COL-SA1920		2.0	0.009			Central intermediary metabolism
COL-SA0678		2.3	0.023			DNA metabolism
COL-SA1241	*recG*	2.5	0.028			DNA metabolism
COL-SA1492	*nth*	3.0	0.009			DNA metabolism
COL-SA1493		2.7	0.045			DNA metabolism
COL-SA1523	*recQ*	2.0	0.007			DNA metabolism
COL-SA1931		2.5	0.016			DNA metabolism
COL-SA0395		2.5	0.001			Energy metabolism
COL-SA0453		2.5	0.008			Energy metabolism
COL-SA1745	*pyk*	2.1	0.014			Energy metabolism
COL-SA1794		2.2	0			Energy metabolism
COL-SA2273	*fdhD*	3.6	0.001			Energy metabolism
COL-SA2553		2.6	0.02			Energy metabolism
COL-SA2482	*fabG*	2.5	0.048			Fatty acid and phospholipid metabolism
COL-SA0085		2.0	0.011			Protein fate
COL-SA1555		2.4	0.039			Protein fate
COL-SA1795	*pepA1*	2.1	0.049			Protein fate
COL-SA2007		2.0	0.013			Protein fate
COL-SA2463	*pepA2*	2.0	0.012			Protein fate
COL-SA1369	*rpmG*	2.0	0.019			Protein synthesis
COL-SA2189		2.0	0.013			Regulatory functions
COL-SA0504		2.3	0.013			Transport and binding proteins
COL-SA0687		3.0	0.023			Transport and binding proteins
COL-SA1114		2.8	0.001			Transport and binding proteins
COL-SA2410		3.2	0.031			Transport and binding proteins
COL-SA2411		2.7	0.006			Transport and binding proteins
COL-SA2572		2.2	0			Transport and binding proteins
COL-SA2721	*nixA*	2.0	0.013			Transport and binding proteins
COL-SA2724		2.5	0.013			Transport and binding proteins
COL-SA2725		2.0	0.003			Transport and binding proteins
III. Downregulation (10 min)-upregulation (20 min)						
COL-SA2515	*gntK*	−3.3	0.033	2.3	0.031	Energy metabolism
COL-SA2516	*gntR*	−3.7	0.014	2.8	0.042	Regulatory functions
COL-SA2662		−3.1	0.009	2.5	0.014	Regulatory functions
COL-SA2471		−2.0	0.045	2.4	0.041	Transport and binding proteins

Continued on following page

TABLE 1—*Continued*

Group and ORF[b]	Gene	10 min		20 min		Functional class
		n-Fold change	*P* value	*n*-Fold change	*P* value	
IV. Downregulation (10 min)-no change (20 min)						
COL-SA1364	*thrB*	−2.0	0.017			Amino acid biosynthesis
COL-SA1596	*aroK*	−2.4	0.01			Amino acid biosynthesis
COL-SA1650	*nadD*	−2.0	0.011			Biosynthesis of cofactors, prosthetic groups, and carriers
COL-SA1819	*ribE*	−2.2	0.003			Biosynthesis of cofactors, prosthetic groups, and carriers
COL-SA1820	*ribD*	−2.0	0.001			Biosynthesis of cofactors, prosthetic groups, and carriers
COL-SA2083	*thiE*	−3.1	0.007			Biosynthesis of cofactors, prosthetic groups, and carriers
COL-SA2084	*thiM*	−2.7	0.01			Biosynthesis of cofactors, prosthetic groups, and carriers
COL-SA2085	*thiD*	−3.2	0			Biosynthesis of cofactors, prosthetic groups, and carriers
COL-SA2122	*coaA*	−2.0	0.011			Biosynthesis of cofactors, prosthetic groups, and carriers
COL-SA2616		−2.0	0.014			Biosynthesis of cofactors, prosthetic groups, and carriers
COL-SA0255		−2.3	0.018			Cell envelope
COL-SA2298		−2.3	0.029			Cell envelope
COL-SA2407		−2.2	0.003			Cell envelope
COL-SA2408		−2.4	0.023			Cell envelope
COL-SA2443		−2.5	0.017			Cell envelope
COL-SA2526		−2.3	0.003			Cell envelope
COL-SA2689	*icaA*	−3.3	0.015			Cell envelope
COL-SA2690	*icaD*	−4.2	0.008			Cell envelope
COL-SA2691	*icaB*	−3.6	0.005			Cell envelope
COL-SA2692	*icaC*	−3.6	0.01			Cell envelope
COL-SA1396	*fmtC*	−2.0	0.044			Cell envelope; cellular processes
COL-SA0665		−2.2	0.036			Cell envelope; transport and binding proteins
COL-SA0935	*dltA*	−2.4	0.021			Cellular processes
COL-SA1824	*arsC*	−2.5	0.016			Cellular processes
COL-SA2712	*drp35*	−5.5	0.034			Cellular processes
COL-SA1328	*glnR*	−3.8	0.044			Cellular processes; regulatory functions
COL-SA2256		−2.4	0.022			Cellular processes; regulatory functions
COL-SA1535	*srrA*	−2.2	0.002			Cellular processes; regulatory functions; regulatory functions; signal transduction
COL-SA1534	*srrB*	−2.4	0.005			Cellular processes; regulatory functions; signal transduction
COL-SA1823	*arsB*	−3.3	0.006			Cellular processes; transport and binding proteins
COL-SA2437	*bcr*	−2.7	0.005			Cellular processes; transport and binding proteins
COL-SA0312	*nanA*	−4.5	0.043			Central intermediary metabolism
COL-SA0315		−2.6	0.001			Central intermediary metabolism
COL-SA0860	*nuc*	−2.9	0.009			DNA metabolism
COL-SA1357		−2.7	0.049			DNA metabolism
COL-SA0251	*bglA*	−2.8	0.017			Energy metabolism
COL-SA0598		−7.1	0.001			Energy metabolism
COL-SA1123	*pyc*	−2.1	0			Energy metabolism
COL-SA1783	*acs*	−4.2	0.028			Energy metabolism
COL-SA1784	*acuA*	−3.6	0.006			Energy metabolism
COL-SA1785	*acuC*	−3.5	0.002			Energy metabolism
COL-SA2527		−4.4	0.002			Energy metabolism
COL-SA2545	*sdhB*	−2.5	0.003			Energy metabolism
COL-SA2664	*manA*	−2.6	0.032			Energy metabolism
COL-SA0214		−2.8	0.005			Fatty acid and phospholipid metabolism
COL-SA0962		−2.2	0.002			Fatty acid and phospholipid metabolism
COL-SA1776		−2.0	0.006			Fatty acid and phospholipid metabolism
COL-SA1005	*pepF*	−2.5	0.035			Protein fate
COL-SA1509		−5.2	0.003			Purines, pyrimidines, nucleosides, and nucleotides
COL-SA1969	*purB*	−2.0	0.04			Purines, pyrimidines, nucleosides, and nucleotides
COL-SA2276		−2.2	0.008			Purines, pyrimidines, nucleosides, and nucleotides
COL-SA0403		−4.3	0.005			Regulatory functions
COL-SA1107		−2.6	0.026			Regulatory functions
COL-SA1552	*malR*	−8.6	0.022			Regulatory functions
COL-SA2086		−3.1	0.011			Regulatory functions
COL-SA2378		−2.5	0.004			Regulatory functions
COL-SA2546		−2.8	0.021			Regulatory functions
COL-SA2030	*scrR*	−2.0	0.022			Regulatory functions
COL-SA2147		−2.2	0.035			Regulatory functions
COL-SA0254		−2.2	0.008			Transport and binding proteins
COL-SA0264		−2.9	0.006			Transport and binding proteins

Continued on following page

TABLE 1—*Continued*

Group and ORF[b]	Gene	10 min		20 min		Functional class
		n-Fold change	*P* value	*n*-Fold change	*P* value	
COL-SA0311		−3.1	0.036			Transport and binding proteins
COL-SA0566	*nupC*	−3.6	0.003			Transport and binding proteins
COL-SA0666		−2.3	0.021			Transport and binding proteins
COL-SA0701		−2.1	0.015			Transport and binding proteins
COL-SA0720		−2.3	0.037			Transport and binding proteins
COL-SA0722		−2.1	0.011			Transport and binding proteins
COL-SA0788		−2.5	0.044			Transport and binding proteins
COL-SA1018		−2.6	0.014			Transport and binding proteins
COL-SA1108		−2.9	0.026			Transport and binding proteins
COL-SA1109		−3.0	0.024			Transport and binding proteins
COL-SA1110		−3.1	0.005			Transport and binding proteins
COL-SA1111		−2.6	0.02			Transport and binding proteins
COL-SA1319	*glpF*	−9.0	0			Transport and binding proteins
COL-SA1367		−2.7	0.045			Transport and binding proteins
COL-SA1427		−2.2	0.014			Transport and binding proteins
COL-SA1728		−2.0	0.003			Transport and binding proteins
COL-SA1743		−2.1	0.015			Transport and binding proteins
COL-SA2335		−2.7	0.019			Transport and binding proteins
COL-SA2340	*gltS*	−4.3	0.024			Transport and binding proteins
COL-SA2356		−3.1	0.037			Transport and binding proteins
COL-SA2441		−4.1	0.035			Transport and binding proteins
COL-SA2442		−2.2	0.044			Transport and binding proteins
COL-SA2525		−3.0	0			Transport and binding proteins
COL-SA2636		−3.4	0.005			Transport and binding proteins
COL-SA0088		−2.0	0.016			Transport and binding proteins
COL-SA0501		−2.5	0.001			Transport and binding proteins
COL-SA1979		−4.8	0.034			Transport and binding proteins
COL-SA0175		−2.4	0.024			Transport and binding proteins; signal transduction
COL-SA0178		−4.1	0.034			Transport and binding proteins; signal transduction
COL-SA0250		−2.5	0.008			Transport and binding proteins; signal transduction
COL-SA0402		−3.5	0.009			Transport and binding proteins; signal transduction
COL-SA1775		−2.0	0.006			Transport and binding proteins; signal transduction
COL-SA2146		−3.5	0.01			Transport and binding proteins; signal transduction
COL-SA2316		−4.4	0.005			Transport and binding proteins; signal transduction
COL-SA2663		−4.5	0.003			Transport and binding proteins; signal transduction
V. Downregulation (10 min)-downregulation (20 min)						
COL-SA1329	*femC*	−2.8	0.022	−2.2	0.024	Cell envelope
COL-SA2566		−2.4	0.001	−3.6	0.005	Cell envelope; transport and binding proteins
COL-SA2347		−3.5	0.002	−2.4	0	Cellular processes; transport and binding proteins
COL-SA2348		−4.2	0.006	−2.1	0.01	Cellular processes; transport and binding proteins
COL-SA0215		−4.1	0.005	−6.4	0.024	Energy metabolism
COL-SA1661		−2.4	0.005	−2.5	0.003	Fatty acid and phospholipid metabolism
COL-SA1662		−3.0	0.01	−2.3	0.021	Fatty acid and phospholipid metabolism
COL-SA0093		−4.1	0.002	−3.7	0.038	Transport and binding proteins
COL-SA2632	*cudT*	−5.3	0.016	−2.6	0.029	Transport and binding proteins
VI. No change (10 min)-upregulation (20 min)						
COL-SA1977	*pheA*			2.2	0.003	Amino acid biosynthesis
COL-SA1181	*arcB*			26.2	0.004	Amino acid biosynthesis; energy metabolism
COL-SA1887	*hemG*			2.3	0	Biosynthesis of cofactors, prosthetic groups, and carriers
COL-SA1888	*hemH*			2.0	0.004	Biosynthesis of cofactors, prosthetic groups, and carriers
COL-SA1168	*efb*			3.1	0.038	Cell envelope
COL-SA1183				5.6	0.001	Cell envelope
COL-SA0245	*lytS*			2.0	0.008	Cell envelope; cellular processes; regulatory functions; signal transduction
COL-SA0246	*lytR*			2.5	0	Cell envelope; cellular processes; regulatory functions; signal transduction
COL-SA0099	*sirA*			2.4	0.05	Cell envelope; transport and binding proteins
COL-SA0193				2.6	0.041	Cell envelope; transport and binding proteins
COL-SA0799				3.5	0	Cell envelope; transport and binding proteins
COL-SA2451				5.1	0.049	Cell envelope; transport and binding proteins

Continued on following page

TABLE 1—*Continued*

Group and ORF[b]	Gene	10 min		20 min		Functional class
		n-Fold change	*P* value	*n*-Fold change	*P* value	
COL-SA2476				3.1	0.024	Cell envelope; transport and binding proteins
COL-SA2173				2.1	0.008	Cellular processes
COL-SA1173	*hlY*			3.3	0.003	Cellular processes
COL-SA1178				2.4	0.044	Cellular processes
COL-SA2006				3.2	0.05	Cellular processes
COL-SA0122				3.3	0.003	Cellular processes; transport and binding proteins
COL-SA1976				2.5	0.004	Central intermediary metabolism
COL-SA0106				4.6	0.029	Central intermediary metabolism; energy metabolism
COL-SA1304				2.6	0.002	DNA metabolism
COL-SA0204	*pflB*			25.1	0.028	Energy metabolism
COL-SA0205	*pflA*			33.2	0.04	Energy metabolism
COL-SA0308				2.5	0.006	Energy metabolism
COL-SA1094	*cydA*			8.4	0.009	Energy metabolism
COL-SA1095	*cydB*			9.0	0.008	Energy metabolism
COL-SA1182	*arcC*			15.4	0.004	Energy metabolism
COL-SA2618	*ldh*			2.2	0.003	Energy metabolism
COL-SA2622	*fdaB*			2.0	0.004	Energy metabolism
COL-SA0121	*deoD*			3.9	0.019	Purines, pyrimidines, nucleosides, and nucleotides
COL-SA2634	*nrdG*			5.2	0.019	Purines, pyrimidines, nucleosides, and nucleotides
COL-SA2635	*nrdD*			6.0	0.002	Purines, pyrimidines, nucleosides, and nucleotides
COL-SA0179				2.0	0.023	Regulatory functions
COL-SA2325				2.5	0.045	Regulatory functions
COL-SA2732				3.6	0.002	Regulatory functions
COL-SA0194				2.3	0.019	Transport and binding proteins
COL-SA0195				2.3	0.008	Transport and binding proteins
COL-SA0217				6.7	0.007	Transport and binding proteins
COL-SA0946				2.0	0.047	Transport and binding proteins
COL-SA1096				2.8	0	Transport and binding proteins
COL-SA1952				2.8	0.006	Transport and binding proteins
COL-SA2472				3.0	0.025	Transport and binding proteins
COL-SA2514	*gntP*			2.0	0.047	Transport and binding proteins
COL-SA0104				4.3	0.04	Transport and binding proteins; cellular processes
COL-SA0105				4.0	0.047	Transport and binding proteins; cellular processes
COL-SA0224				2.5	0.005	Transport and binding proteins; signal transduction
VII. No change (10 min)-downregulation (20 min)						
COL-SA2627	*betA*			−3.4	0.001	Cellular processes
COL-SA2628	*betB*			−3.2	0.001	Cellular processes
COL-SA0008	*hutH*			−2.4	0.03	Energy metabolism
COL-SA1741	*icd*			−2.6	0.005	Energy metabolism
COL-SA1742	*gltA*			−2.7	0.012	Energy metabolism
COL-SA0213				−2.0	0.044	Fatty acid and phospholipid metabolism
COL-SA0987	*fabH*			−2.1	0.006	Fatty acid and phospholipid metabolism

[a] The genes were grouped based on their regulation directions upon 10- and 20-min exposures. Note that genes belonging to the functional classes of "hypothetical proteins," "hypothetical proteins-conserved," and "unknown function" are not included.
[b] Prefix indicates the name of the *S. aureus* strain.

change in the expression level of this gene at 20 min of exposure might indicate that this lesion was already repaired by the base excision pathway at that time. 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.

Of particular interest was the finding that several genes directly associated with the virulence of *S. aureus* were included in group II. Specifically, the Cap5C protein encoded by COL-SA0138 is involved in capsular polysaccharides synthesis, which enhances staphylococcal virulence by impeding phagocytosis (45). COL-SA1522 encodes an elastin binding protein which promotes bacterial adherence to extracellular matrix and thus the colonization of host tissues during infection (49). Furthermore, COL-SA1179 and -SA1180 reportedly encode exotoxins which are involved in food poisoning and toxic shock syndrome (22, 59). This finding is congruent with the previous outcome that hydrogen peroxide induces several virulence factor-related genes in *P. aeruginosa* (46). Indeed, virulence-related enzymes are involved in microbial defense systems against oxidants by damaging phagocytes and/or impairing oxidants (40). Further, it was previously revealed that virulence factors scavenge reactive oxygen species (7, 8, 32). Consequently, the possibility that the virulence-related genes mentioned above, such as *cap5C* and COL-SA1178 to -SA1180, contributed to cellular oxidative defense against hydrogen peroxide in *S. aureus* should not be excluded.

Group IV comprised 132 gene that exhibited mRNA level decreases at 10 min of exposure and no significant changes at 20 min of exposure (Table 1). This group represents the largest portion of the statistically significant 343 genes in our study. One of the most distinctive features of group IV was that 40 out of the 132 genes belonged to the functional class of "transport and binding proteins." These genes are primarily related to permeases and ATP-binding cassette (ABC) proteins. As presented in Fig. 2, this functional class consists of a total of 288 genes in *S. aureus*; thus, 14% of its genes were repressed at 10 min in response to hydrogen peroxide exposure. This finding possibly implies that membrane components of *S. aureus* were altered and that active and/or facilitated transport through the cell membrane was initially attenuated upon exposure to hydrogen peroxide. Furthermore, the finding that most of these genes exhibited no expression level changes at 20 min of exposure indicates that the transport system of *S. aureus* was restored, which might be linked to the resumption of growth.

Particularly important was the finding that many of the genes in the class of "transport and binding proteins" were also members of the "signal transduction" class (Table 1). Intriguingly, the genes were all involved in the bacterial phosphoenolpyruvate:sugar phosphotransferase system, which mediates the uptake and phosphorylation of carbohydrates and controls metabolism in response to their availability (25). The system is composed of several types of proteins; however, the genes here exclusively encode the carbohydrate-specific enzymes IIA and IIB and/or the membrane permease IIC, which recognizes and transports the sugar molecules (25). Besides, we found that COL-SA0403 and -SA2147, genes adjacent to COL-SA0402 and -SA2146, code for enzymes belonging to the BglG family of transcriptional antiterminators that regulate the expression of bacterial genes and operons, whose products are required for utilization of phosphoenolpyruvate:sugar phosphotransferase system carbohydrates (21). Hence, the repression of these genes can directly deteriorate carbohydrate uptake and subsequent metabolism in *S. aureus*, which might be associated with the growth arrest effect of hydrogen peroxide.

To our surprise, the repression of the intercellular adhesion locus (*icaADBC*), which is associated with the virulence activity of *S. aureus*, was found in group IV. The *icaADBC* locus mediates polysaccharide intercellular adhesion (PIA) production in *S. aureus* and *Staphylococcus epidermidis*, which leads to cell-cell adhesion and is required for biofilm formation (13, 41, 56). PIA is synthesized by the expression of the *icaADBC* genes, which encode three membrane proteins (IcaA, IcaD, and IcaC) with enzymatic activity and one extracellular protein (IcaB) (17). Prior studies demonstrated that PIA production is involved in the pathogenesis of *S. epidermidis* (50, 51) and is also induced by subinhibitory concentrations of certain antibiotics (48). Considering these previous conclusions, it was striking that hydrogen peroxide-driven oxidative stress repressed the transcription of the *icaADBC* locus in our study. However, our result may propose the possibility that hydrogen peroxide insult attenuated biofilm formation, which depends on the activity of the *icaADBC* locus.

Table 1 also shows that many genes related to primary metabolic pathways, including the classes of "energy metabolism" and "fatty acid and phospholipid metabolism," were repressed in response to hydrogen peroxide. This phenomenon might reflect general changes in cellular physiology and a metabolic repression as a result of oxidative damage. Related to this finding, we also discovered that a number of genes in the class of "biosynthesis of cofactors, prosthetic groups, and carriers" were repressed. All these genes are responsible for the synthesis of various cofactors such as nicotinamide, pantothenate, riboflavin, and thiamine, which are essential for many enzymatic reactions in respiration. Therefore, it can be postulated that the repression of these genes interfered with part of respiratory metabolic pathways, which may be associated with the growth inhibition seen upon 10 min of exposure.

Group VI consisted of 68 genes whose expression levels increased only in response to 20 min of exposure (Table 1). The most dominant class was "transport and binding proteins," which possessed one-fourth of the genes in group VI. Moreover, many of the genes in this class encode proteins that convey cations and iron-carrying compounds. In particular, COL-SA0099 (*sirA*), -SA0104, and -SA0105 aroused our attention because they are involved in iron uptake system in *S. aureus*. First, COL-SA0104 and -SA0105 code for siderophore (iron-chelating compound) biosynthesis proteins. Siderophore-mediated iron uptake is one of the most important mechanisms that bacteria use to acquire iron from the environment (57). Second, the SirA protein encoded by COL-SA0099 is involved in iron-siderophore import in *S. aureus* (14). Iron metabolism is coordinately regulated with oxidative stress defenses because iron promotes the formation of hydroxyl radicals, which indiscriminately damage all cellular components (62). Further, superoxide, generated during the process of oxygen reduction, releases free iron from iron-sulfur proteins, thus increasing the levels of intracellular free iron (30). Supporting this hypothesis, COL-SA0665 and -SA0666 of group IV, iron compound transport proteins, were repressed upon 10 min of exposure to hydrogen peroxide. Consequently, our results may indicate that the iron uptake system was attenuated to prevent further oxidative damage and/or was initially inactivated by the increased concentration of intracellular free iron resulting from the oxidative damage, but the uptake resumed subsequently for cellular growth after the normal iron level was restored.

In relation to iron metabolism, we also found genes responsible for heme synthesis and iron storage in group VI. COL-SA1887 (*hemG*) and -SA1888 (*hemH*) code for proteins involved in heme synthesis; in particular, HemH catalyzes the final step of heme biosynthesis, which involves the insertion of ferrous iron into protoporphyrin IX (19). Heme is essential for respiration and defense against oxygen intermediates because heme compounds are cofactors for cytochromes and catalases (19). Consistent with our finding, it was previously demonstrated that control of heme biosynthesis is attuned more to oxidative stress than to iron levels and HemH is induced by hydrogen peroxide-driven oxidative stress in *Salmonella enterica* (19). Next, the protein encoded by COL-SA0799 and -SA1952 was homologous to a ferritin family protein. This outcome is intriguing because ferritins are the major iron storage proteins that contribute to scavenging intracellular iron, which lessens cellular oxidative damage, as discussed above (42). Hence, this result suggests that the induction of these genes possibly helped protect against hydrogen peroxide-caused oxidative stress by controlling intracellular iron levels.

TABLE 2. Transcript level comparison of *S. aureus* genes involved in anaerobic metabolic pathways between real-time PCR analysis and microarray analysis[a]

Gene	mRNA level change		Primer sequence (sense, antisense)
	Microarray	Real-time PCR	
SA0204 (*pflB*)	25.1	107.3 ± 50.5	5′-AAAGCAGGCGTTATTACTGAAAGC-3′ 5′-CGTCAATACCTACACCACCGATAG-3′
SA0205 (*pflA*)	33.2	81.8 ± 38.1	5′-TGACAAACATATTAGATTGACAGGAAAGC-3′ 5′-ATCATCAGAATAACCAGGCACAAGG-3′
SA1094 (*cydA*)	8.4	18.5 ± 8.1	5′-TCTCAGCCTTCTTCATTACTTCAG-3′ 5′-ACAAATGCCATCGTCATACCG-3′
SA1095 (*cydB*)	9.0	24.8 ± 8.0	5′-AGTACCAGGTTCAATAGCACTGATTATG-3′ 5′-TGCCAAGAATACTACAGACCAAGC-3′
SA1181 (*arcB*)	26.2	47.4 ± 16.6	5′-AGACTTTTCACGACAAGAGGTAG-3′ 5′-TGCCATCATACATTCCACCAAG-3′
SA1182 (*arcC*)	15.4	22.3 ± 9.0	5′-GAAAATCACCTCAAGAACAACTC-3′ 5′-TGTAATTGATAGCCGATGTAAGC-3′
SA2618 (*ldh*)	2.2	3.4 ± 1.3	5′-GGTGAGCATGGTGATACTGAAC-3′ 5′-TCCATAGTATGTTGACCCTTTAGC-3′
SA2634 (*nrdG*)	5.2	6.6 ± 1.7	5′-GTTGACGGTGAAGGAGTAAGATG-3′ 5′-AATCCAGTCCATACCCAAATTGTC-3′
SA2635 (*nrdD*)	6.0	10.4 ± 3.5	5′-CATCTAATGGACAGACACCTTTTG-3′ 5′-ATGTCATAGTTCGGATCTTGCG-3′
16S rRNA			5′-GCGAAGAACCTTACCAAATC-3′ 5′-CCAACATCTCACGACACG-3′

[a] The results shown are the means of three biological replicates with three technical replicates each (± the standard error) for each gene.

However, the reason that these genes were upregulated only upon 20 min of exposure awaits further investigation.

In Table 1, another notable finding was the presence of COL-SA0245 (*lytS*) and -SA0246 (*lytR*) in group VI. The *lytS* and *lytR* genes, whose products are members of the two-component regulatory family of proteins, are involved in the control of autolysis by affecting murein hydrolase activity, which is important in the biological processes of antibiotic resistance, cell division, cell-to-surface adhesion, and biofilm formation (4, 20, 27, 39). Specifically, a *lytS* mutant strain exhibits an increased propensity for spontaneous lysis and an increased rate of penicillin- and Triton X-100-induced lysis in *S. aureus* (4, 27), whereas a *lytR* mutant strain shows defective cell division and attenuated autolytic activity (11). In conjunction with this finding, we also observed that the *scdA* gene of group I, which is immediately upstream of the *lytSR* genes and important for staphylococcal cell division (5), showed much stronger expression (10-fold) upon 20 min of exposure. Consequently, our data suggest that the induction of *lytSR* might be involved in the regulation of cell division, which apparently occurred more vigorously upon 20 min of exposure in our study.

Table 1 also shows that several genes related to pathogenesis of *S. aureus* were present in group VI. For example, COL-SA1168 (*efb*) encodes a virulence factor that binds to both the complement C3b and fibrinogen, inhibits complement activation, and blocks opsonophagocytosis (33). Further, COL-SA1173 (*hly*) and -SA2006 are likely associated with alpha-toxins, which cause membrane damage to many types of mammalian cells (6). It should be emphasized that many of the virulence-related genes of *S. aureus* were differently regulated in response to oxidative stress; that is, such virulence-related genes as *cap5C*, COL-SA1179, and -SA1180 of group II were induced only at 10 min, whereas others including *efb*, *hly*, and COL-SA2006 of group VI were induced only at 20 min. On the other hand, the *icaADBC* genes were downregulated upon exposure to hydrogen peroxide.

The last and perhaps most striking result is that a number of genes of group VI encode proteins involved in anaerobic metabolism, most of which belonged to the functional class of "energy metabolism." Table 1 shows that COL-SA0204 (*pflB*), -SA0205 (*pflA*), -SA1094 (*cydA*), -SA1095 (*cydB*), -SA1181 (*arcB*), -SA1182 (*arbC*), -SA2618 (*ldh*), -SA2634 (*nrdG*), and -SA2635 (*nrdD*) were classified into that category. Note that the expression level changes of all these genes were also validated by using quantitative real-time PCR analysis. Table 2 shows that our microarray results were corroborated by real-time PCR analysis, which provides independent verification of transcript level changes of the genes discussed here.

First, the *pflBA* genes, which exhibited 25- and 33-fold increases in transcription levels upon 20 min, respectively, code for enzymes homologous to pyruvate formate-lyases that catalyze the nonoxidative dissimilation of pyruvate to acetyl coenzyme A and formate when *E. coli* grows under oxygen-limiting conditions (54). The proteins encoded by *arcBC* are responsible for the arginine deiminase pathway, which enables arginine-dependent anaerobic growth (36). Further, the *ldh* gene codes for a protein that shares considerable homology to

a lactate dehydrogenase that converts pyruvate to lactate in *E. coli* under anaerobic conditions (29). The *nrdDG*-encoded enzymes are the class III ribonucleotide reductases that are responsible for the synthesis of deoxyribonucleotides needed for DNA synthesis under oxygen-limiting conditions (38).

Moreover, the *cydAB* genes, which together encode cytochrome *d* oxidase, were strongly induced by eight- and ninefold, respectively, upon 20 min of exposure. Cytochrome *d* oxidase catalyzes the last step of oxygen respiration and prevails under oxygen-limiting conditions (26). Interestingly, it was speculated that cytochrome *d* oxidase is required under conditions of environmental stress and may have crucial roles in cellular physiology other than acting as an oxidase (12). Further, prior studies revealed that cytochrome *d* oxidase plays an imperative part in cellular protection against oxidative stress, at least under microaerobic growth conditions, by showing that mutation or deletion of the genes encoding the enzyme increases sensitivity to oxidative stress in *E. coli* and *Azotobacter vinelandii* (18, 23, 35). It was also suggested that the ability of cytochrome *d* oxidase to reduce dioxygen to water might minimize the generation of reactive oxygen species (15). Therefore, the result that the *cydAB* genes were strongly induced upon exposure to hydrogen peroxide strengthens the confidence of the prior assignments about the role of cytochrome *d* oxidase in oxidative protection processes.

As mentioned above, in addition to this oxidative protective role of cytochrome *d* oxidase, it is also known to be associated with microaerobic dioxygen respiration (15). That is, the transcription of the *cydAB* genes is activated when oxygen becomes limiting (26). Further, Alexeeva et al. proposed that the rapid consumption of oxygen by cytochrome *d* oxidase may contribute to the activity of pyruvate formate-lyase under microaerobic conditions by demonstrating that increased expression of genes coding for cytochrome *d* oxidase and pyruvate formate-lyase is coordinated in *E. coli* (1). This hypothesis might account for the phenomenon in our study that the pyruvate formate-lyase genes (*pflBA*) were induced upon 20 min of exposure, in chorus with the strong expression of the cytochrome *d* oxidase genes (*cydAB*).

Consequently, our result described here possibly implies that *S. aureus* experienced an oxygen-limiting state in response to hydrogen peroxide-driven oxidative stress. Supporting this possibility is the finding that genes responsible for fermentative metabolism (*pflBA*, *arcBC*, *ldh*, and *nrdGD*), as well as genes encoding cytochrome *d* oxidase (*cydAB*), were upregulated upon 20 min of exposure. Further, our observation with respect to the transcription level changes of the *pflBA* and *ldh* genes is congruent with the previous result that in *E. coli pfl* and *ldhA* are induced by more than 10-fold and 2-fold, respectively, by shifting the culture condition from an aerobic to a microaerobic state (47). Therefore, it seems that *S. aureus* underwent similar conditions upon exposure to hydrogen peroxide in our study. Notably, the finding that these genes were significantly induced only upon 20 min of exposure suggests that fermentative or microaerobic respiration, which had not been initially activated, was stimulated afterward in response to oxidative stress. Moreover, despite the activation of fermentative metabolism, which provides significantly less energy, *S. aureus* was able to resume growing at the same rate as untreated controls (Fig. 1). Considering the fact that our cultures were provided with sufficient aeration for growth, the reason for this phenomenon is obscure. However, the possibility that the cells might strive to avoid further cytotoxicity arising from reactive oxidants produced during normal oxygen respiration should not be excluded. Indeed, this speculation is in line with the outcome of a prior study by Sabra et al. which demonstrated that *Pseudomonas aeruginosa* prefers microaerobic conditions for growth and for the formation of some of its virulence factors under oxidative stress (52). Most strikingly, a similar phenomenon was also observed in mammalian cells. That is, several species of parasites, such as *Schistosoma mansoni*, *Angiostrongylus cantonensis*, and *Dirofilaria immitis*, show a reduction of their aerobic respiration along their developmental cycles on vertebrate blood, relying on fermentation to achieve their energy requirements (44). Further, the study proposed that the arrest of respiration constitutes an adaptation to avoid the toxic effects of reactive oxygen species (44).

Conclusions. In this study, we demonstrated for the first time how oxidative stress-induced genes are related and regulated in *S. aureus* by using whole-genome microarrays. Moreover, we showed how the transcriptome profile of *S. aureus* was shifted during its cellular response to oxidative stress, which involved growth inhibition and resumption. In summary, we revealed that DNA repair and virulence factor genes were selectively upregulated between growth inhibition and resumption. We also found that growth inhibition was accompanied by the repression of many membrane function-related genes; however, the majority of these genes returned to normal transcription levels during growth resumption. Further, we showed the induction of iron uptake- and storage-related genes, which was accompanied by growth recovery, following the repression of iron compound-transporting genes. Notably, we discovered the induction of fermentative metabolism-related genes and cytochrome *d* oxidase genes while the cells returned to normal growth. These results suggest that *S. aureus* might undergo an oxygen-limiting state upon exposure to hydrogen peroxide. Further, we propose that this phenomenon benefited *S. aureus* by preventing further cytotoxicity arising from reactive oxygen species produced during oxygen respiration. To our knowledge, this is the first study demonstrating the activation of fermentative metabolism under oxidative stress in *S. aureus*. Hence, we are currently exploring whether the induction of the responsible genes helps protect against toxic oxidants in *S. aureus* and how this event is linked to growth resumption.

Nucleotide sequence accession number. The data discussed in this publication have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) and are accessible through GEO Series accession number GSE3415. Additionally, the data can be accessed at http://www.umbi.umd.edu/%7Ecbr/lab_web/home.htm.

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