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GENOMICS AND PROTEOMICS

Microarray analysis of toxicogenomic effects of triclosan on *Staphylococcus aureus*

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Abstract For the first time, a genome-wide transcriptional analysis was performed to elucidate the cellular response of *Staphylococcus aureus* to triclosan. Our results indicate that the effects of triclosan are widespread on metabolism, affecting many vital cellular processes. Triclosan downregulated the transcription of genes involved in virulence factor and energy metabolism such as amino acid, carbohydrate, lipid transport, and metabolism, while multidrug resistance genes, coenzyme transport, and metabolism and transcription genes were upregulated. Furthermore, triclosan downregulated the transcription of genes encoding major lipid metabolism enzymes such as 3-hydroxyacyl-CoA dehydrogenase, acetyl-CoA acetyltransferase, acetyl-CoA synthetase, and acetyl-CoA carboxylase, which all play essential roles in *S. aureus* lipid metabolism. It is interesting to note that the expression of the enoyl-ACP reductase gene, *fab*I, was not changed after exposure of *S. aureus* with 0.05 μM triclosan at 10 and 60 min in our study. This work also implies that triclosan may kill *S. aureus* by interfering with its ability to form cell membranes. Another important implication of our result is that *S. aureus* may generate resistance factors under triclosan stress.

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Introduction

Triclosan is one of the most common antimicrobial agents on the market today. Its widespread use spans from hospital environment to facial cleansers and liquid hand soaps due to its bactericidal activity. Because triclosan is frequently used in applications that simultaneously contact both humans and bacteria, it is important to understand the differential effects on each so that its efficacy can be understood and even optimized. There have been several reports related to the exposure of triclosan on humans, but there are few reports that delineate its specific activity on bacteria and few that concern *Staphylococcus aureus*, a pathogen of significant importance and a target of triclosan (Heath et al. 1999). The most of triclosan uses are in consumer products that are eventually disposed of down sink drains. Wastewater treatment plants cannot remove triclosan from water, so large quantities of triclosan are continuously discharged into local waterways. Numerous studies have detected triclosan in streams and rivers (Barber et al. 2006; Heidler and Halden 2007; Heidler et al. 2006). In a U.S. Geological Survey study of several organic wastewater contaminants in U.S. streams, triclosan was one of the most frequently detected compounds, and at some of the highest concentrations observed (Kolpin et al. 2002; Loraine and Pettigrove 2006).

Many recent studies have raised serious concerns that triclosan may promote the emergence of bacteria that are resistant to antibiotics (Russell 2002a, b; Weber and Rutala 2006). One concern is that bacteria will become resistant to antibacterial products like triclosan, rendering those antimicrobial products useless to those who truly need them, such as people with compromised immune systems. Triclosan kills bacteria in a similar way as antibiotics. Triclosan resistance may contribute to reduced susceptibility to clinically important antimicrobials due to either cross-resistance or coresistance mechanisms (Yazdankhah et al. 2006). It creates an environment where mutated bacteria that are resistant to triclosan are more likely to survive and reproduce.

Earlier studies show that triclosan inhibits lipid biosynthesis in *Escherichia coli*, probably by action upon enoyl reductase (FabI), which was screened in triclosan-resistant mutants of *E. coli* K12 strain AG100 using LB agar plate with triclosan (McMurry et al. 1998). Furthermore, enoyl-acyl carrier protein (ACP) reductase, which catalyzes the last step in each cycle of fatty acid elongation, is a promising target because it plays a key role in the regulation of the lipid metabolism pathway. In several previous studies, triclosan was found to inhibit FabI, the enoyl-ACP reductase of *E. coli*, *Pseudomonas aeruginosa*, and *S. aureus* (Heath et al. 2000; Hoang and Schweizer 1999).

Recently, DNA microarray approaches have been increasingly used to elucidate the cellular responses of bacteria to antimicrobial compounds in attempts to identify patterns that could suggest the mode of action (MOA) of novel antibacterial agents. In our previous studies, we investigated the MOA in global genome expression in *S. aureus* and *P. aeruginosa* by using whole-genome microarrays and characterized the critical role played by genes in viability and during treatment of antimicrobial compounds such as hypochlorite, peracetic acid, and hydrogen peroxide (Chang et al. 2005a, b; Chang et al. 2006a, b; Small et al. 2007a, b). In this study, to our knowledge, for the first time, we show that the global transcription response of *S. aureus* to triclosan includes the downregulation of genes encoding lipid metabolism proteins and genes encoding carbohydrate transport and metabolism proteins. Based on this result, this study will help elucidate the mechanisms by which triclosan kills microbes and facilitate the design of more effective antimicrobials.

Materials and methods

Bacterial strains and growth conditions

In this study, we used *S. aureus* NCTC 8325 obtained from the Network on Antimicrobial Resistance in *S. aureus* (NARSA). As previously described (Chang et al. 2006a, b), we initiated and maintained *S. aureus* cultures at 37°C with shaking at 250 rpm using sterilized Luria–Bertani (LB) broth. For growth inhibition, triclosan was dissolved in DMSO and various concentrations of triclosan (Aldrich Chemical, St. Louis, MO, USA) were added immediately after the optical density at 600 nm reached the early logarithmic phase (0.8). For growth inhibition, 0.015625 mg/l (0.05 μM) of triclosan (Aldrich Chemical, St. Louis, MO, USA) was used for the microarray study and added immediately after OD_{600} reached 0.8. OD_{600} was measured by using Lambda 25 spectrophotometer (PerkinElmer, MA, USA). Note that the pH of *S. aureus* cultures was around 7.0 at 37°C after the exposure (Dukan et al. 1997).

RNA isolation

Total RNA was isolated after 10 and 60 min incubation with and without (control) triclosan using the RiboPure-Bacteria kit (Ambion, Austin, TX, USA) (Chang et al. 2006a, b). RNA quality was determined using both Lambda 25 spectrophotometer (PerkinElmer, MA, USA) and RNA 6000 Nano LabChip with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

cDNA synthesis, labeling, hybridization, staining, and scanning

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, Santa Clara, CA, USA).

Affymetrix *S. aureus* GeneChip analysis

The arrays were scanned with the Affymetrix GeneChip Scanner 3000. To analyze the array data, the GeneChip Operating Software (GCOS) v. 1.2 (Affymetrix, Santa Clara, CA, USA) and GeneSpring GX v. 7.3 (Agilent Technologies, Santa Clara, CA, USA) were utilized with the following parameters: α_1=0.04; α_2=0.06; τ=0.015; target signal=500. Fold changes were calculated as the ratio between the signal averages of five biological controls (untreated) and five biological experimental (triclosan-treated) for 10 and 60 min exposures.

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 Table 2. The housekeeping gene 16S rRNA was used as an endogenous control. Real-time PCR was performed by employing the iCycler iQ Real-time PCR Detection System with iScript cDNA Synthesis Kit and IQ SYBR Green Supermix (BioRad Laboratories, Hercules, CA, USA). For each gene, five biological replicates with five technical replicates each were employed. Reaction mixtures were initially incubated for 3 min at 95.0°C, followed by 40 cycles of 10 svh 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, Hercules, CA, USA). 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 vs 16S rRNA gene was utilized to determine transcript level changes.

Results

Growth inhibition by triclosan

To determine the sublethal inhibitory effect of triclosan on *S. aureus*, we first exposed the exponentially growing cells to triclosan. In Fig. 1, we demonstrate that 0.05 μM triclosan caused a growth inhibition for about 10 min. Note that the minimum inhibitory concentration (MIC) of triclosan on *S. aureus* is reportedly 0.015 μM (Lambert 2004). Figure 1 shows the effects of different concentrations of triclosan on the growth rate of *S. aureus*. These data indicated that the growth rate was inhibited by triclosan. Thus, in this study, to better understand how *S. aureus* initially responds to triclosan, we chose the rate of inhibition at 10 and 60 min exposure times with 0.05 μM triclosan.

Microarray analysis of *S. aureus* response

To investigate the changes in transcriptome profiles in response to triclosan, we conducted five independent microarray experiments in the absence (control) and the presence (experimental) of 0.05 μM triclosan upon 10 and 60 min exposures (see Fig. 1). To further identify genes with statistically marked changes in expression levels, we applied the following criteria to each of the 10 and 60 min control–experimental microarray data sets: (1) a p value for a t test should be ≤0.05, (2) an absolute fold change in transcript level should be ≥2, and (3) a gene should have a presence or marginal call (Affymetrix 2004) from 50% or more replicates on both the experimental and control replicate sets. Of the 2,892 genes in the *S. aureus* genome, 707 genes showed statistical significance based on a one-way ANOVA. We found that mRNA levels of 122 genes of *S. aureus* were significantly altered in response to triclosan by twofold or more. The raw data of 2,892 control (10 for 60 min) and experimental (after 10 and 60 min exposure) genes has been deposited in the National Center for Biotechnology Information's (NCBI) Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) and is accessible through GEO Series accession number GSE8861

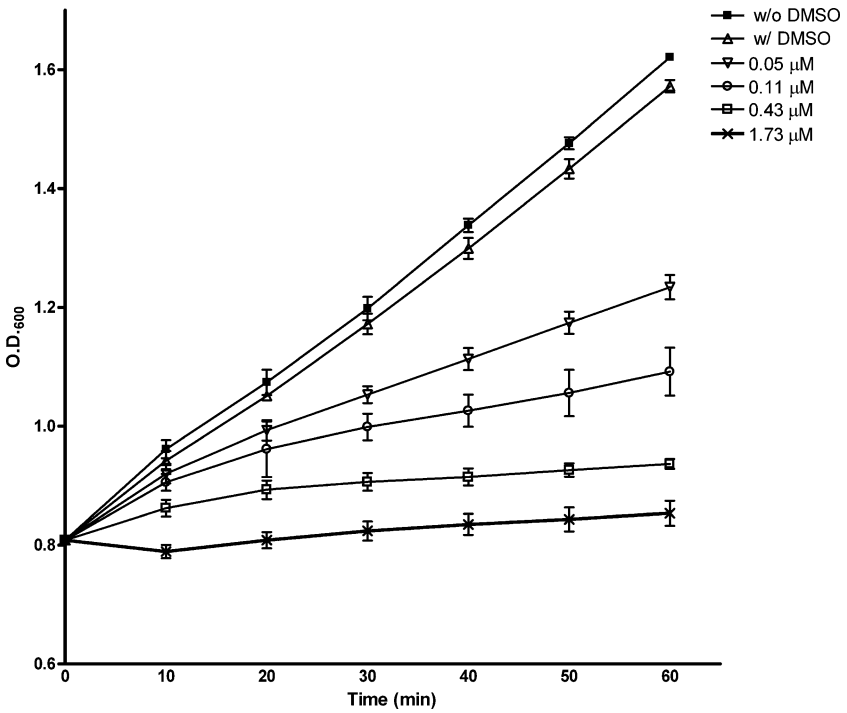

Fig. 1 *S. aureus* growth (optical density at 600 nm) after treatment with triclosan: without DMSO (*closed squares*), with DMSO (*open triangles*), 0.05 μM (*inverted open triangles*), 0.11 μM (*open circles*), 0.43 μM (*open squares*), and 1.73 μM (*X*). The growth rate (μ) was calculated during the exponential phase of the cells with triclosan: without DMSO, with DMSO, 0.05, 0.11, 0.43, and 1.73 μM triclosan. The results are the mean of triplicate experiments; the *error bars* represent the standard deviation

(http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GSE8861&targ=self&form=html&view=quick).

To test the validity of the microarray data, we further performed real-time PCR analysis on 10 genes with a range of fold changes (−5.79- to 3.65-fold, which were specifically involved in the pathogenesis or metabolism of *S. aureus*). Table 2 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.

Functional classifications analysis

Functional classifications of the responding genes are provided in Fig. 2. To examine how genes with transcript level changes are distributed with regard to their functions, we further classified these 122 genes according to the Gene Classification based on COG functional categories in the genome of NCBI (http://www.ncbi.nlm.nih.gov/sites/entrez?db=genome&cmd=Retrieve&dopt=Overview&list_uids=19213).

In Fig. 2, there are 122 genes included in the group of functional unknown and general function predicted only. Figure 2 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. The interesting findings included that (1) the genes of carbohydrate transport and metabolism were downregulated at both 10 and 60 min; (2) the genes of lipid transport and metabolism were also downregulated at both 10 and 60 min; (3) the genes of coenzyme transport, metabolism, and transcription were upregulated at 60 min; and (4) the number of genes of the amino acid transport and metabolism, carbohydrate transport and metabolism, cell cycle control, cell wall/membrane/envelope biogenesis, defense mechanism, energy production and conversion, inorganic ion transport and metabolism, lipid transport and metabolism, posttranslational modification, and secondary metabolites biosynthesis classes were downregulated after 60 min.

To further identify genes with similar transcription patterns during the time course, we categorized the 94 genes into 4 groups on the basis of their transcription directions. Group I was comprised of genes downregulated in response to both 10 and 60 min exposures. Genes in group II were downregulated only after 10 min response. Genes in group III were upregulated at 60 min. Group IV contained genes downregulated at 60 min. Figure 3 and Table 1 display the number of genes (94) of groups I, II, III, and IV in each functional class. Note that the genes whose function is unknown are excluded in Fig. 3 and Table 1. Table 1 displays the individual genes with the functional class of each group, their fold changes, and p values in response to 10 and 60 min exposures. To assess how genes with transcript level changes are distributed with regard to their functions, we classified the differently regulated 94 genes according to the categories described in the genome

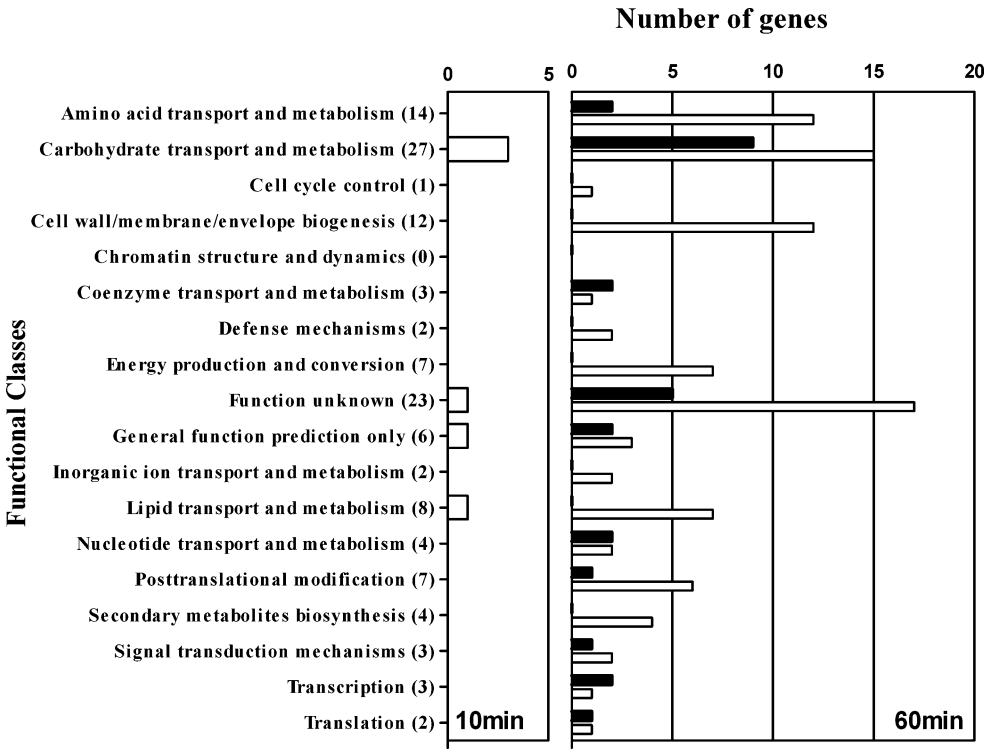

Fig. 2 Functional classification of genes with statistically significant increase (*closed squares*) and decrease (*open squares*) in the mRNA level upon 10 and 60 min exposures (a total of 122 genes). The *number in parenthesis* represents the total number of genes within the genome in each functional class

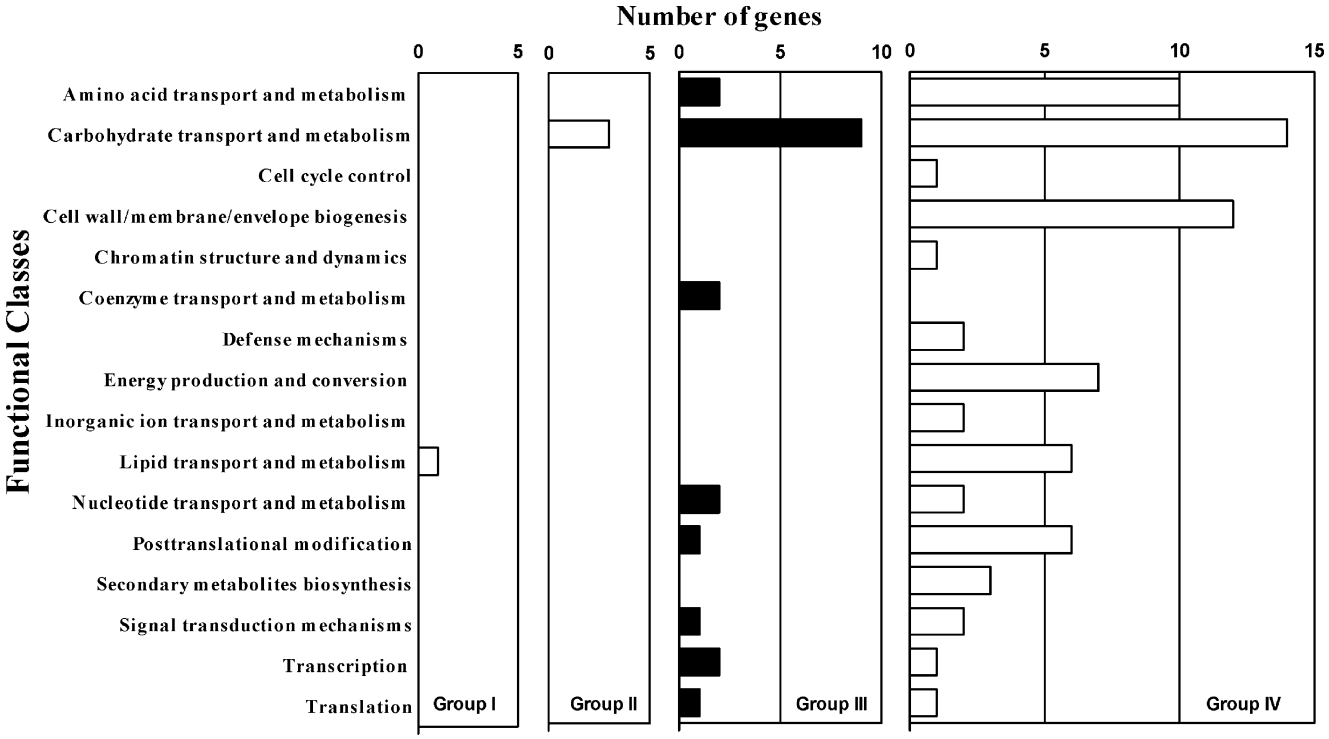

Fig. 3 The number of genes within groups I through IV in each functional class. Functional classification of genes with statistically significant increase (*closed squares*) and decrease (*open squares*) in the mRNA level upon 10 and 60 min exposures (a total of 94 genes). Note that the functional classes of general function predicted only and function unknown are not included in this figure

of NCBI. In Fig. 3, we display the number of differentially regulated genes in each functional class. In Fig. 3, group I implies that the lipid transport and metabolism-related genes were downregulated at 10 and 60 min. In Fig. 3, group II indicates that carbohydrate transport and metabolism also were downregulated in 10 min. In group III of Fig. 3, there were some upregulated genes from "carbohydrate transport and metabolism" and "amino acid transport and metabolism". The other interesting points in group III were that "coenzyme transport and metabolism", "nucleotide transport and metabolism", "signal transduction mechanisms", "transcription", and "translation" classes were upregulated. Figure 3 implies that the functional classes of group IV in general contained more downregulated genes at 60 min. In particular, the functional classes of "amino acid transport and metabolism", "carbohydrate transport and metabolism", "function unknown", "cell wall/membrane/envelope biogenesis", and "energy production and conversion" had significantly more downregulated genes at 60 min. Furthermore, the genes were downregulated at 60 min in the classes of "lipid transport and metabolism", "posttranslational modification, protein turnover, chaperones", "secondary metabolites biosynthesis, transport and catabolism", and "defense mechanisms". This result suggests that the functional class profiles were notably different between 10 and 60 min. All of 94 gene functions (Tables 1 and 2) are discussed below.

Discussions

Group I: genes downregulated upon 10 and 60 min exposures Group I was comprised of one gene downregulated in response to both 10 and 60 min exposures. SA0225 encodes acyl-CoA dehydrogenase in the class of "lipid transport and metabolism". Entry into the beta-oxidation cycle requires the action of acyl-CoA dehydrogenase, the first enzyme in the sequence. This result implies that an acyl-CoA dehydrogenase may be involved in the early response of *S. aureus* to triclosan (see also Table 2).

Group II: genes downregulated upon 10 min exposures Three genes of group II were downregulated only after 10 min response. It is interesting to note that SA0653–SA0655 (*fruA* and *B*) putatively encodes proteins involved in the fructose transport and metabolism. In this study, we also found two genes, *lrgA* and *B*, which were downregulated by 2.3- and 2.8-fold, respectively. There is a relatively recent study indicating the inhibition of extracellular murein hydrolase activity and increased tolerance to penicillin (Bayles 2000). As some bacterial murein hydrolases have the potential to cause cell lysis, which effects the regulation of critical importance to the cell. The function of the *lrgAB* gene products is hypothesized to be analogous to that of a bacteriophage-encoded antiholin, which inhibits the formation of murein hydrolase transport channels in the bacterial

Table 1 Lists of the *S. aureus* genes of each group, their fold changes, *p* values, and functional classes in response to 10 and 60 min exposures to triclosan

ORF no.	10 min		60 min		Description	Gene symbol	Functional class
	Fold change	*p* value	Fold change	*p* value			
Group I: downregulation (10 min)–downregulation (60 min)							
SA0225	−2.018	0.00277	−13.31	0.000457	Acyl-CoA dehydrogenase		Lipid transport and metabolism
Group II: downregulation (10 min)							
SA0653	−2.18	0.0056			Transcription repressor of fructose operon		Carbohydrate transport and metabolism
SA0654	−2.083	0.00706			Fructose 1-phosphate kinase	*fruB*	Carbohydrate transport and metabolism
SA0655	−2.616	0.00706			Fructose-specific permease	*fruA*	Carbohydrate transport and metabolism
Group III: upregulation (60 min)							
SA0254			2.535	0.00192	Transcription regulator GntR family		Transcription
SA0255			2.946	0.00681	PTS beta-glucoside-specific enzyme II, ABC component		Signal transduction
SA0256			3.052	0.00216	6-phospho-beta-glucosidase	*bglA*	Carbohydrate transport and metabolism
SA0294			2.285	0.00271	Branched-chain amino acid uptake carrier		Amino acid transport and metabolism
SA0354			2.228	0.0211	30S ribosomal protein S18	*rpsR*	Translation, ribosomal structure and biogenesis
SA0373			2.595	0.00246	Xanthine phosphoribosyltransferase	*xprT*	Nucleotide transport and metabolism
SA0374			2.34	0.00179	Xanthine permease	*pbuX*	Nucleotide transport and metabolism
SA0478			2.265	0.000116	Putative pyridoxine (vitamin B_6) biosynthetic enzyme		Coenzyme transport and metabolism
SA0653			2.76	0.00227	Transcription repressor of fructose operon		Carbohydrate transport and metabolism
SA0654			2.138	0.00384	Fructose 1-phosphate kinase	*fruB*	Carbohydrate transport and metabolism
SA0665			2.292	0.00955	Coenzyme PQQ synthesis homologue		Posttranslational modification, protein turnover, chaperones
SA0666			2.401	0.00908	6-pyruvoyl tetrahydrobiopterin synthase homologue		Coenzyme transport and metabolism
SA1239			2.039	0.00908	Branched-chain amino acid carrier protein	*braB*	Amino acid transport and metabolism
SA1972			2.347	0.00179	Multidrug transporter		Carbohydrate transport and metabolism
SA2053			2.054	0.00179	Glucose uptake protein homolog		Carbohydrate transport and metabolism
SA2167			2.952	0.00917	PTS system, sucrose-specific IIBC component	*scrA*	Carbohydrate transport and metabolism
SA2203			2.518	0.00216	Multidrug resistance protein		Carbohydrate transport and metabolism
SA2293			3.321	0.0204	Gluconate permease	*gntP*	Carbohydrate transport and metabolism
SA2294			3.973	0.0156	Gluconokinase	*gntK*	Carbohydrate transport and metabolism
SA2295			3.312	0.0302	Gluconate operon transcriptional repressor	*gntR*	Transcription

Table 1 (continued)

ORF no.	10 min		60 min		Description	Gene symbol	Functional class
	Fold change	*p* value	Fold change	*p* value			
Group IV: downregulation (60 min)							
SA0122			−2.007	0.000105	Acetoinreductase	*butA*	Secondary metabolites biosynthesis, transport and catabolism
SA0131			−3.178	0.00757	Purine nucleoside phosphorylase	*pnp*	Nucleotide transport and metabolism
SA0144			−3.318	2.84E-05	Capsular polysaccharide synthesis enzyme Cap5A	*capA*	Cell wall/membrane/ envelope biogenesis
SA0145			−3.35	7.26E-05	Capsular polysaccharide synthesis enzyme Cap5B	*capB*	Cell wall/membrane/envelope biogenesis
SA0146			−3.114	4.06E-05	Capsular polysaccharide synthesis enzyme Cap8C	*capC*	Cell wall/membrane/envelope biogenesis
SA0147			−2.673	0.000204	Capsular polysaccharide synthesis enzyme Cap5D	*capD*	Cell wall/membrane/envelope biogenesis
SA0148			−2.677	0.000143	Capsular polysaccharide synthesis enzyme Cap8E	*capE*	Cell wall/membrane/envelope biogenesis
SA0149			−2.784	4.45E-05	Capsular polysaccharide synthesis enzyme Cap5F	*capF*	Cell wall/membrane/envelope biogenesis
SA0150			−2.557	0.000115	Capsular polysaccharide synthesis enzyme Cap5G	*capG*	Cell wall/membrane/envelope biogenesis
SA0151			−2.513	7.26E-05	Capsular polysaccharide synthesis enzyme O-acetyl transferase Cap5H	*capH*	Cell wall/membrane/envelope biogenesis
SA0152			−2.176	7.26E-05	Capsular polysaccharide synthesis enzyme Cap5I	*capI*	Cell wall/membrane/envelope biogenesis
SA0153			−2.093	0.000337	Capsular polysaccharide synthesis enzyme Cap5J	*capJ*	Cell wall/membrane/envelope biogenesis
SA0186			−2.461	0.0284	Sucrose phosphotransferase enzyme II		Carbohydrate transport and metabolism
SA0187			−2.482	0.0292	Transcription regulator		Transcription
SA0214			−2.008	0.0226	Hexose phosphate transport protein	*uhpT*	Carbohydrate transport and metabolism
SA0223			−8.904	8.12E-05	Acetyl-CoA acetyltransferase homologue		Lipid transport and metabolism
SA0224			−12.79	0.000216	3-Hydroxyacyl-CoA dehydrogenase		Lipid transport and metabolism
SA0226			−8.479	0.000219	Acid-CoA ligase		Secondary metabolites biosynthesis, transport and catabolism
SA0227			−6.585	0.000118	Conserved hypothetical protein		Lipid transport and metabolism
SA0231			−2.325	0.00035	Flavohemoprotein		Energy production and conversion
SA0258			−2.459	0.00168	Probable ribokinase	*rbsK*	Carbohydrate transport and metabolism
SA0259			−2.405	0.00384	Ribose permease	*rbsD*	Carbohydrate transport and metabolism
SA0260			−2.542	0.00492	Ribose transporter RbsU		Carbohydrate transport and metabolism
SA0276			−2.329	3.49E-05	Similar to diarrheal toxin		Cell cycle control, cell division, chromosome partitioning
SA0319			−4.493	0.0146	Conserved hypothetical protein		Carbohydrate transport and metabolism

Table 1 (continued)

ORF no.	10 min		60 min		Description	Gene symbol	Functional class
	Fold change	*p* value	Fold change	*p* value			
SA0320			−4.712	0.0148	PTS fructose-specific enzyme IIBC component		Carbohydrate transport and metabolism
SA0321			−4.466	0.0197	Transcription antiterminator BglG family		Carbohydrate transport and metabolism
SA0330			−2.039	0.00339	Ribosomal-protein-serine *N*-acetyltransferase		Translation, ribosomal structure, and biogenesis
SA0381			−2.186	0.000313	Conserved hypothetical protein		Carbohydrate transport and metabolism
SA0605			−2.393	0.034	Dihydroxyacetone kinase		Carbohydrate transport and metabolism
SA0606			−2.157	0.036	Conserved hypothetical protein		Carbohydrate transport and metabolism
SA0755			−2.673	7.41E-05	General stress protein 170		Posttranslational modification, protein turnover, chaperones
SA0820			−2.608	0.0148	Glycerophosphoryl diester phosphodiesterase	*glpQ*	Energy production and conversion
SA0835			−2.022	0.0197	ClpB chaperone homologue	*clpB*	Posttranslational modification, protein turnover, chaperones
SA0891			−2.123	9.38E-05	Ferrichrome ABC transporter		Inorganic ion transport and metabolism
SA1244			−2.007	0.0141	Dihydrolipoamide succinyltransferase	*odhB*	Energy production and conversion
SA1245			−2.437	0.0131	2-Oxoglutarate dehydrogenase E1	*odhA*	Energy production and conversion
SA1432			−2.476	0.00296	Conserved hypothetical protein		Inorganic ion transport and metabolism
SA1434			−2.503	0.000642	Acetyl-CoA carboxylase (biotin carboxylase subunit) accC homolog		Lipid transport and metabolism
SA1435			−2.386	0.0016	Acetyl-CoA carboxylase (biotin carboxyl carrier subunit) accB homolog		Lipid transport and metabolism
SA1436			−2.387	0.00168	Conserved hypothetical protein		Amino acid transport and metabolism
SA1437			−2.396	0.00111	Conserved hypothetical protein		Amino acid transport and metabolism
SA1542			−2.449	0.000789	Glycerophosphoryl diester phosphodiesterase		Energy production and conversion
SA1553			−2.154	0.0082	Formyltetrahydrofolate synthetase	*fhs*	Nucleotide transport and metabolism
SA1554			−3.595	0.0201	Acetyl-CoA synthetase	*acsA*	Lipid transport and metabolism
SA1556			−3.316	0.00437	Acetoin utilization protein	*acuC*	Chromatin structure and dynamics
SA1609			−2.676	0.0461	Phosphoenolpyruvate carboxykinase	*pckA*	Energy production and conversion
SA1882			−2.113	0.00166	Sensor protein KdpD	*kdpD*	Signal transduction mechanisms
SA1883			−2.011	0.000429	KDP operon transcriptional regulatory protein KdpE	*kdpE*	Signal transduction mechanisms
SA2082			−2.234	0.000352	Urease gamma subunit	*ureA*	Amino acid transport and metabolism
SA2083			−2.097	7.26E-05	Urease beta subunit	*ureB*	Amino acid transport and metabolism

Table 1 (continued)

ORF no.	10 min		60 min		Description	Gene symbol	Functional class
	Fold change	*p* value	Fold change	*p* value			
SA2084			−2.019	7.93E-05	Urease alpha subunit	*ureC*	Amino acid transport and metabolism
SA2114			−3.433	0.0292	PTS system, arbutin-like IIBC component	*glvC*	Carbohydrate transport and metabolism
SA2125			−2.012	0.00179	Formiminoglutamase		Amino acid transport and metabolism
SA2149			−3.382	0.000337	ABC transporter ATP-binding protein		Defense mechanisms
SA2150			−4.03	0.000332	Conserved hypothetical protein		Defense mechanisms
SA2235			−2.184	0.000636	Glycine betaine/carnitine/choline ABC transporter opuCC	*opuCC*	Cell wall/membrane/ envelope biogenesis
SA2236			−2.392	6.08E-05	Probable glycine betaine/ carnitine/choline ABC transporter opuCB	*opuCB*	Amino acid transport and metabolism
SA2237			−2.401	0.000115	Glycine betaine/carnitine/choline ABC transporter opuCA	*opuCA*	Amino acid transport and metabolism
SA2336			−2.864	4.54E-05	ATP-dependent Clp proteinase chain clpL	*clpL*	Posttranslational modification, protein turnover, chaperones
SA2405			−2.037	0.0141	Choline dehydrogenase	*betA*	Amino acid transport and metabolism
SA2406			−2.659	0.00172	Glycine betaine aldehyde dehydrogenase gbsA	*gbsA*	Energy production and conversion
SA2430			−2.382	2.84E-05	Zinc metalloproteinase aureolysin	*aur*	Amino acid transport and metabolism
SA2434			−3.276	0.0127	Fructose phosphotransferase system enzyme fruA homolog		Carbohydrate transport and metabolism
SA2435			−2.757	0.0287	Mannose-6-phosphate isomerase		Carbohydrate transport and metabolism
SA2459			−2.019	0.0022	Intercellular adhesion protein A	*icaA*	Cell wall/membrane/envelope biogenesis
SA2490			−2.496	0.000113	*N*-hydroxyarylamine *O*-acetyltransferase		Secondary metabolites biosynthesis, transport and catabolism
SACOL 1874			−2.003	0.000849	Epidermin leader peptide processing serine protease EpiP	*epiP*	Posttranslational modification, protein turnover, chaperones
SACOL 1877			−2.302	0.000105	Epidermin biosynthesis protein EpiB	*epiB*	Posttranslational modification, protein turnover, chaperones
SACOL 1878			−3.473	3.26E-05	Lantibiotic epidermin precursor E	*epiA*	Posttranslational modification, protein turnover, chaperones

The genes were grouped based on their regulation directions upon 10 and 60 min exposures. Ninety-four out of 122 genes were used in this table. Note that the functional classes of "general function predicted only" and "function unknown" are not included in this table.

membrane. These transport channels are proposed to be targets for the bactericidal effects of penicillin. The expression of the *lrgAB* operon is known to be temporally regulated with maximal expression occurring during the transition into the stationary phase. This expression is thought to be partially responsible for the growth-phase-dependent sensitivity of *S. aureus* to penicillin-induced lethality.

Group III: genes upregulated upon 60 min exposure Twenty genes in group III showed increased expression levels at 60 min. Table 1 indicates that one of the characteristics of group III was the upregulation of nine genes in the functional class of "carbohydrate transport and metabolism". Among these genes, SA0255 (*bglA*) encodes 6-phospho-beta-glucosidase that catalyses the conversion of 6-phospho-beta-D-glucosyl-(1, 4)-D-glucose and H_2O to D-glucose-6-phosphate and D-glucose. These genes were involved in the bacterial phosphoenolpyruvate/sugar phosphotransferase system (PTS), which mediates the uptake and phosphorylation of carbohydrates and controls

Table 2 Transcript level comparison of virulence factor genes of *S. aureus* between real-time PCR and microarray analyses

Gene	mRNA level change with real-time PCR	mRNA level change with microarray	Sense primer sequence	Antisense primer sequence
SA0223	−5.79 (±0.31)	−8.904	5′-AAC GAG CCC ACA AAC AAT CCA ACC-3′	5′-TTT GAA CCG ACC ATC ACG TTG TGC-3′
SA0224	−5.79 (±0.84)	−12.79	5′-TTT GAA CCG ACC ATC ACG TTG TGC-3′	5′-TGT GCC TGG CTT TGT CGC AAA TAG-3′
SA0225	−5.04 (±1.04)	−2.018	5′-TGT GCC TGG CTT TGT CGC AAA TAG-3′	5′-ATG CTT AAC TTG TGT TGC TCG GCG-3′
SA0227	−1.95 (±0.15)	−6.585	5′-ATG CTT AAC TTG TGT TGC TCG GCG-3′	5′-CTT GAA ACA GTC GCT GAA CGC CAA-3′
SA0654 (*fru*B)	+2.70 (±0.31)	+2.138	5′-CTT GAA ACA GTC GCT GAA CGC CAA-3′	5′-ATC GAC GCC ATC TTG TTC TGG TCT-3′
SA0835 (*clp*B)	−1.74 (±0.10)	−2.022	5′-TTC GGC ATT AGA GCG TCG TTT CCA-3′	5′-ATC AGA CAA TTC AGC GGC AGC AAC-3′
SA1434	−2.05 (±0.40)	−2.503	5′-ATC AGA CAA TTC AGC GGC AGC AAC-3′	5′-TGC GAG CAG AAC GTG ATT TGG TTG-3′
SA1435	−2.14 (±0.20)	−2.386	5′-TGC GAG CAG AAC GTG ATT TGG TTG-3′	5′-ACG CTG TCA CTT TAC CTG GTG TTG-3′
SA2203	+3.65 (±0.23)	+2.518	5′-ACG CTG TCA CTT TAC CTG GTG TTG-3′	5′-CTC GAA TCA TTT GGC TGC ACA CAG-3′
16S rRNA[a]	1.00	1.00	5′-GCG AAG AAC CTT ACC AAA TC-3′	5′-CCA ACA TCT CAC GAC ACG-3′
SA0869 (*fab*I)	1.02 (±0.05)	1.068	5′-ACA TCA GCG CCT ACA GCT TCA T-3′	5′-GCC CAT TAC GTT GTA GTT CGG CAT-3′

The results are the mean of three biological replicates with three technical replicates each for each gene.
[a] 16S rRNA was used as the housekeeping gene.

metabolism in response to their availability (Gosset 2005). Besides, we found that SA0654 (*fruB*) putatively codes for a fructose 1-phosphate kinase involved in carbohydrate metabolism that employs the phosphoenolpyruvate (PEP)/sugar PTS, which generates fructose-l-phosphate as the cytoplasmic product of the PTS-catalyzed phosphorylation reaction (see also Table 2). Fructose-l-phosphate kinase then converts the product of the PTS reaction to fructose-1,6-bisphosphate (Mitchell et al. 1993). SA2167 codes for PTS system-related sucrose-specific IIBC component (*scrA*) that regulates the expression of bacterial genes and operons whose products are required for the utilization of PTS carbohydrates (Fux et al. 2004). Hence, the repression of these genes can directly deteriorate carbohydrate uptake and the subsequent metabolism in *S. aureus*. Despite the downregulation of SA0654 (*fruB*) and SA0653 of group II, which is related to the operon of fructose metabolism, these genes, especially in group III, were upregulated upon exposure to triclosan. It is interesting to note that two of the nine genes, SA1972 and SA2203, code for proteins associated with multidrug transport and resistance (see also Table 2). In particular, the proteins encoded by SA2293 (*gntP*), SA2294 (*gntK*), and SA2295 (*gntR*), organized in a predicted operon, and SA0254 are involved in gluconate operon. SA2053 codes for a glucose uptake protein homolog which is followed by a glucose dehydrogenase gene. Group III in Table 1 also shows that two genes in the functional class of "amino acid transport and metabolism" were upregulated at 60 min. SA0294 and SA1239 (*braB*) encode the Na(+)-coupled carrier for branched-chain amino acids in *S. aureus*. The *braB* gene codes for a protein that restores growth at low leucine concentration and Na(+)-dependent leucine transport activity to *P. aeruginosa* PAO3536 defective in the transport of branched-chain amino acids (Hoshino et al. 1990).

The proteins encoded by SA0478, SA0665, and SA0666, putative pyridoxine (vitamin B_6) biosynthetic enzyme, coenzyme pyrroloquinoline quinone (PQQ) synthesis homologue and 6-pyruvoyl tetrahydrobiopterin synthase homologue, respectively, are involved in coenzyme transport and metabolism. In particular, PQQ is an important cofactor of bacterial dehydrogenases including glucose dehydrogenase, linking the oxidation of many different compounds to the respiratory chain. This suggested that glucose uptake and metabolism are involved in triclosan-induced damage, followed most likely by phosphorylation via glucokinase.

Triclosan upregulates the expression of SA2423 encoding the clumping factor B (ClfB), which binds fibrinogen (Ni Eidhin et al. 1998; Table 1, group III).

Group IV: genes downregulated upon 60 min exposure Seventy genes in group IV were downregulated at 60 min. As shown in Table 1, the most dominant class was "carbohydrate transport and metabolism". The PTS 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 (Gosset 2005). Despite that SA2167, sucrose-specific IIBC component (*scrA*), is upregulated in group III, most of the carbohydrate transport and metabolism of group IV were downregulated in this study, responsible for PTS-related proteins, such as SA0320, SA0186, SA2114(*glvC*), SA2434, and SA2435. We also observed genes related to primary metabolism that mainly belonged to the functional classes of "energy metabolism", and "purines, pyrimidines, nucleosides, and nucleotides". For example, SA0131, SA1553, SA1609, SA1244, and SA1245 (*pnp*, *fhs*, *pckA*, *odhB*, and *odhA*) were downregulated at 60 min. Oxoglutarate dehydrogenase (*odhA*) is a key control point in the citric acid cycle. It is inhibited by its products, succinyl-CoA and NADH. A high-energy charge in the cell is also inhibited.

One of the characteristics of group IV was the downregulation of 12 genes belonging to the functional class of "cell wall/membrane/envelope biogenesis". In particular, genes related to cell wall synthesis were distinctive: (1) SA0144–SA0153 (*capABCDEFGHIJ*) were downregulated at 60 min, which is homologous to the capsular polysaccharide synthesis enzyme (cap) operon, which in turn is essential for virulence; (2) IcaA encoded by SA2459, a virulence factor, which synthesizes a polysaccharide, poly-*n*-succinyl-beta-1, 6 glucosamine (PNSG) during infection. PNSG is critical to biofilm elaboration, allowing bacteria to adhere to one another, and may also promote adherence to other molecules, such as ECM components (Cramton et al. 1999); (3) SA2235 (*opuCC*) encodes glycine betaine/carnitine/choline ABC transporter OpuCC. The operon is preceded by a potential SigB-dependent promoter. An *opuC*-defective mutant was generated by the insertional inactivation of the *opuCA* gene. A *Listeria monocytogenes* mutant was impaired for growth at high osmolarity in brain–heart infusion broth and failed to grow in a defined medium (Fraser et al. 2000). We conclude that the *opuC* operon in *S. aureus* encodes an ABC-compatible solute transporter which is capable of transporting L-carnitine and which plays an important role in osmoregulation and downregulated by triclosan in *S. aureus*. Therefore, this result and the repression of cell wall synthesis genes in group IV imply that triclosan might suppress the synthesis of some of the virulence factors in *S. aureus*.

Several of the operons that were upregulated in biofilms have been found to be important in acid tolerance in other bacterial species. It is interesting to note that we found that multiple genes, urease gamma, beta, and alpha (SA2082–SA2084; *ureABC*), were downregulated in *S. aureus* by triclosan.

SA0321 codes for the transcription antiterminator BglG family, which is involved in the positive control of the utilization of different sugars by transcription antitermination. For some of these regulatory proteins, it has been demonstrated that antitermination is exerted by binding to a conserved RNA sequence, partially overlapping the transcription terminator, and thus preventing transcription termination (Bardowski et al. 1994).

SACOL1874, SACOL1877, and SACOL1878 encode lantibiotic epidermin biosynthesis proteins, *epiP*, *epiB*, and *epiA*. Lantibiotics such as epidermin, nisin, Pep5, and subtilin are ribosomally synthesized as prepeptides and posttranslationally are converted into the mature peptide. Epidermin is synthesized as a 52-amino-acid precursor peptide, EpiA, which is posttranslationally modified and processed to the mature 22-amino-acid peptide antibiotic.

Members of the two-component regulatory system *kdpD*/*kdpE*, SA1882–SA1883, are involved in the regulation of the kdp operon. KdpD may function as a membrane-associated protein kinase that phosphorylates kdpE in response to environmental signals.

ClpB chaperone homologue (SA0835) and ATP-dependent Clp protease chain clpL (SA2336), encoded by *clpBL*, contain two conserved ATP-binding domains and are upregulated by heat shock. In a previous report, the *clpB*, *clpC*, or *clpE* disruption mutants were as tolerant of heat and salt as the wild-type *Lactococcus lactis*. However, *clpB* mutant cells were slightly more resistant to puromycin than wild-type *Lactococcus lactis* (Ingmer et al. 1999).

Another notable finding of group IV was that six genes were involved in lipid transport and metabolism functions (Table 1). Prior studies demonstrated that triclosan inhibits bacterial fatty acid synthesis at the enoyl-acyl carrier protein reductase (FabI) step (Rock and Cronan 1996; Zhang and Cronan 1998). The importance of fatty acid biosynthesis to cell growth and function makes this pathway an attractive target for the development of antibacterial agents. Two important control points in the cycle are the condensing enzymes and the enoyl-ACP reductase (Heath et al. 2000; Heath et al. 1999), and both reactions are targeted by compounds that effectively inhibit fatty acid synthesis. However, our result shows that triclosan downregulates two genes, *accC* and *accB* (SA1434 and SA1435), which are acetyl-CoA carboxylase homologs on fatty acid biosynthesis (see also Table 2). SA0223, SA0224, SA0225, and SA0227 encode acetyl-CoA acetyltransferase homologue, 3-hydroxyacyl-CoA dehydrogenase, and acyl-CoA dehydrogenase, which are involved in fatty acid oxidation. In addition, the acyl-CoA dehydrogenase gene is also downregulated in 10 min in

Table 1, group I (see also Table 2). It is interesting to note that the enoyl-ACP reductase gene, SA0869, was not changed after treatment of triclosan. Our microarray results were corroborated with real-time PCR analysis.

In summary, this paper describes the first genome-wide transcriptional analysis of *S. aureus* response to triclosan. Briefly, our data based on the toxicogenomic analysis showed the following results. First, triclosan resulted in the downregulation of primary metabolism-related and carbohydrate transport and metabolism genes. Most of the catabolism-related genes, including glycerollipid-, sucrose metabolism- and glycolysis-related genes, were downregulated by triclosan. Second, the cap operon, which in turn is essential for virulence, was also downregulated in *S. aureus*. It is intriguing to note that virulence-related genes are involved in microbial defense systems against triclosan.

Third, triclosan downregulated the ClpB chaperone-related genes of *S. aureus*. The *clpB* deletion mutant cells were slightly more resistant to puromycin than wild-type cells (Ingmer et al. 1999). This outcome implies that the resistance of *S. aureus* to triclosan was altered, and furthermore, the resistance through the Clp proteins was downregulated upon exposure to triclosan. That is, *S. aureus* may adopt the presence of triclosan as an environmental signal that triggers the expression of resistance determinants.

S. aureus downregulated the genes involved in fatty acid production and utilization upon exposure to triclosan. Most interestingly, we provided evidence that triclosan resulted in the reduction of the fatty acid metabolism-related genes of *S. aureus*. These genes code for acetyl-CoA acetyltransferase homologue, 3-hydroxyacyl-CoA dehydrogenase, and acyl-CoA dehydrogenase, which all play essential roles in fatty acid metabolism. The inhibition of fatty acid synthesis is the major mode of action of triclosan, killing this pathogen (McMurry et al. 1998) by interfering with its ability to form cell membranes. However, our data also suggest that the reduction of acetyl-CoA acetyltransferase homologue, 3-hydroxyacyl-CoA dehydrogenase, and acyl-CoA dehydrogenase might be involved in the regulation of cell survival, which apparently occurred more vigorously upon 60 than 10 min exposure in our study. That is, triclosan may trigger to decay the fatty acid metabolism of *S. aureus* and parts of the virulence factors, in particular, the cap operon, which in turn is essential for virulence. Another important implication of our result is that *S. aureus* may generate resistance factors, ClpB chaperone-related gene, under triclosan stress. Consequently, we are currently exploring whether the upregulation and/or downregulation of the responsible genes help protect against triclosan in *S. aureus* and how this event is linked to the bacterial resistance and metabolism.

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