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Differential gene expression in *Staphylococcus aureus* exposed to Orange II and Sudan III azo dyes

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Abstract

We previously demonstrated the effects of azo dyes and their reduction metabolites on bacterial cell growth and cell viability. In this report, the effects of Orange II and Sudan III on gene expression profiling in *Staphylococcus aureus* ATCC BAA 1556 were analyzed using microarray and quantitative RT-PCR technology. Upon exposure to 6 μg/ml Orange II for 18 h, 21 genes were found to be differently expressed. Among them, 8 and 13 genes were up- and down-regulated,

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respectively. Most proteins encoded by these differentially expressed genes involve stress response caused by drug metabolism, oxidation, and alkaline shock indicating that *S. aureus* could adapt to Orange II exposure through a balance between up and down regulated gene expression. Whereas, after exposure to 6 μg/ml Sudan III for 18 h, 57 genes were differentially expressed. In which, 51 genes were up-regulated and 6 were down-regulated. Most proteins encoded by these differentially expressed genes involve in cell wall/membrane biogenesis and biosynthesis, nutrient uptake, transport and metabolite, and stress response, suggesting that Sudan III damages the bacterial cell wall or/and membrane due to binding of the dye. Further analysis indicated that all differentially expressed genes encoded membrane proteins were up-regulated and most of them serve as transporters. The result suggested that these genes might contribute to survival, persistence and growth in the presence of Sudan III. Only one gene *msrA*, which plays an important role in oxidative stress resistance, was found to be down-regulated after exposure to both Orange II and Sudan III. The present results suggested that both these two azo dyes can cause stress in *S. aureus* and the response of the bacterium to the stress is mainly related to characteristics of the azo dyes.

Keywords

Staphylococcus aureus; Orange II; Sudan III; Microarray; Gene expression

Introduction

Azo dyes consist of one or more azo groups (R_1–N = N–R_2), which are characterized by a diazotized amine coupled to an amine or a phenol [12, 79]. It is estimated that more than 2000 different azo dyes are ubiquitously used in various industrial sectors including the plastics, textile, paper, food, cosmetics, and pharmaceutical industries [11, 24, 58, 74]. Humans are exposed to these compounds through ingestion, inhalation, or skin contact. Thus the wide application of various azo dyes in foods, drugs and cosmetics, has raised a concern in regard to the impacts of colorants on human health and the environment. It has been demonstrated that some azo dyes are associated with bladder cancer in humans, splenic sarcomas, hepatocarcinomas, and nuclear anomalies in experimental animals, and chromosomal aberrations in mammalian cells [19, 37, 55]. In general, azo dyes are considered as xenobiotic compounds that are very recalcitrant with respect to biodegradation [74].

Nevertheless, more and more microorganisms have been found to be capable of degrading azo dyes under certain conditions [14, 24, 64, 73, 81]. The first step of azo dye degradation is reduction of the azo bond catalyzed by azoreductase, which has recently been reviewed [11]. Orange II (D&C Orange No. 4) is a water-soluble sulfonated dye, while Sudan III (D&C Red No. 17) is a water-insoluble diazo dye. Their chemical structures and properties are shown in Table 1. Both are approved for use in drug and cosmetic products as colorants [49]. There is evidence that water-soluble azo dyes can penetrate through the cell membrane and subsequently be reduced by azoreductase in the cytoplasm [23]. Whereas, for water-insoluble Sudan dyes, their degradation might be extracellular and/or membrane associated processes rather than an intracellular one [81]. It has been demonstrated that *Escherichia coli* is not able to efficiently degrade Sudan I, II, III, IV and Para Red due to lack of membrane

azo reduction enzymes and membrane bound azo dyes are not efficiently transported into the bacterial cells [43, 81].

Human skin is an intricate habitat for a diverse population of microbiota including commensal and pathogenic bacteria contributing to both human health and disease [31]. *Staphylococcus* is one of the most prevalent aerobic genera on human skin. Our previous studies have shown that *Staphylococcus aureus* is capable of degrading both Orange II and Sudan III [57, 73]. In addition, we found that a tetrameric NADPH-dependent flavin azoreductase (Azo1) from *S. aureus* (ATCC 25923) metabolizes Methyl Red (MR), Orange II, Amaranth, Ponceau BS and Ponceau S [10]. No significant effect on cell growth and cell viability of the bacterium was found after exposure to 6 μg/ml Orange II and Sudan III in our previous study [57]. However, very little is known about the effects of the azo dyes on gene expression in *S. aureus*. Here we report genome-wide responses of *S. aureus* to azo dyes assessed by microarray and quantitative RT-PCR assays with differential gene expression profiling of the bacterium in the presence of water soluble Orange II and water insoluble Sudan III.

Materials and methods

Materials

Orange II (4-(2-hydroxy-1-naphthylazo) benzenesulfonic acid sodium salt), Sudan III (1-[4-(phenylazo)phenylazo]-2-naphthol), dimethyl sulfoxide (DMSO), isopropanol, ethanol, chloroform, and acid-washed glass beads (212–300 μm) were purchased from Sigma-Aldrich Co. Stock solutions of Orange II and Sudan III were freshly prepared by dissolving the chemicals in deionized water and DMSO, respectively.

Bacterial strain and culture conditions

S. aureus ATCC BAA 1556 was used for the experiments in this study. The strain was routinely cultured in Brain Heart Infusion (BHI) agar plate media. After 16–18 h of incubation at 37 °C, one colony was picked by a loop and inoculated into a 15-ml centrifuge tube containing 10 ml BHI medium. The culture was incubated in static conditions at 37 °C overnight for use as seed culture. The bacterial seed culture was inoculated into BHI medium with an inoculation ratio of 1 % (v/v), and then 40 ml aliquots of the medium were transferred to 50-ml Falcon centrifuge tubes. Stock solutions of Orange II or Sudan III were added to the BHI medium at final concentrations of 6 μg/ml (each in triplicate, all experiments were triplicate unless otherwise stated). The cultures without azo dyes but with an equal volume of deionized water or DMSO were inoculated with the bacterial strain as controls. The cultures were incubated at 37 °C without agitation. Decolorization of the dyes was monitored by spectrometry. For Orange II, 1 ml samples were collected from the cultures at various points in time, and centrifuged at 10,000×*g* for 3 min. Supernatants of the cultures were assayed by measuring the absorption in a Beckman Coulter DU 800 UV–visible spectrophotometer at 483 nm. For Sudan III, two volumes of absolute ethanol were added to 0.5 ml of the samples collected from the cultures. The mixtures were briefly vortexed, and then the samples were assayed at 500 nm following the procedure described

above for Orange II. After 18 h incubation, the cultures were collected for total RNA isolation as described below.

Total RNA isolation and purification

The cultures were collected by centrifugation at 3200×*g* for 15 min. Cell pellets were washed with diethylpyrocarbonate-treated deionized water (DEPC-treated H_2O, 0.1 %) twice. Each cell pellet was suspended in 1 ml DEPC-treated H_2O and frozen at −70 °C for 20 min. Then acid-washed glass beads (450 mg) were added, and the cells were disrupted at top speed on a VORTEX-GENIE 2 (Scientific Industries, Inc.) at 4 °C for 30 min. The mixtures of disrupted cells and glass beads were transferred to 15-ml centrifuge tube. Then total RNA was extracted following the RNA-Bee™ (Tel-Test, Inc.) reagent procedure. Five ml RNA-Bee and 0.5 ml chloroform were added, mixed and incubated on ice for 15 min followed by centrifugation at 3,200×*g* for 30 min. The upper phase (aqueous layer) was transferred to a clean tube and equal volume of isopropanol was added, mixed gently and stored at −80 °C for 30 min or overnight at −20 °C. RNA precipitation was collected by centrifugation at 4 °C. The pellet was washed with 75 % ethanol (diluted by DEPC-treated H_2O) twice by vortexing, dried by letting it stand on the bench for 5–10 min and dissolved in 100 μl DEPC-treated H_2O. The total RNA was then purified according to the standard protocol provided with an RNeasy mini kit (QIAGEN). Contaminating genomic DNA in the RNA preparations was removed using DNA-free kit (Ambion). DNA removal was confirmed by PCR. The RNA quality and quantity were determined by agarose gel electrophoresis and by Nanodrop 1000 (Thermo Scientific), respectively. DNA-free RNA was stored at −80 °C.

Microarray

The microarray was constructed by MYcroarray Inc. using 3 catalog 3 × 15 K *S. aureus subsp. aureus* USA300 Microarrays. This strain name is referred to as *S. aureus* ATCC BAA 1556 in this study. The complete genome sequence of this strain is available at http://www.ncbi.nlm.nih.gov/nuccore/CP000255. Each 3 × 15 K microarray has three separate arrays composed of 16,320 spots, of which 12,860 spots (~45-mers) for *S. aureus* genes. The remaining 3,460 are control features (spike-in empty spots, positive controls, negative control probes, etc.). There are 5 identical replicates of each *S. aureus* probe such that a total of 2572 *S. aureus* genes are surveyed by each array (http://www.MYcroarray.com). Hybridization image on the slide was scanned using an Axon4000B scanner (Molecular Devices).

Statistical and bioinformatic analysis

The scanned hybridization images were quantified by GenePix Pro Software (version 6.1.0.4). Array data were normalized using scaling normalization to adjust the total or average intensity of each array to be approximately the same [82]. Microarray data analysis was conducted using a FDA microarray software, ArrayTrack [77]. Lists of differentially expressed genes were identified using a two group (Sample vs. Control) *t* test after excluding spots flagged as bad. The criteria of *p* value <0.05 and an absolute relative ratio ≥1.5-fold were applied. Additionally, Venn diagrams were used to examine the overlap of resulting lists of genes differentially expressed between the different sample groups.

The differentially expressed genes were sorted into functional categories based on clusters of orthologous groups (COGs) [45]. Subcellular localizations of proteins, encoded by differentially expressed genes, were predicted using PSORTb ver. 3.0 (http://psort.org) [85].

Validation of microarray data by quantitative real-time PCR (qRT-PCR)

Some genes (including one azoreductase gene-SAUSA300_0545 (*azo1*)) identified by the microarray were then randomly selected for confirmation by qRT-PCR. The special primers were designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), an enhanced web interface to Primer3. The sequences of primers used in this study are listed in Table 2. Total RNA (2.5–4.5 μg) was reverse transcribed into single-stranded cDNA using Transcriptor First-Strand cDNA Synthesis Kit (Roche Diagnostics) with both Anchored-oligo (dT) 18 primer and random hexamer primer, according to the manufacturer's instructions. The reaction mixture in tube was incubated for 10 min at 25 °C, 30 min at 55 °C, 5 min at 85 °C and then placed the tube on the ice. The synthesized cDNA was stored at −20 °C and diluted 1/10 prior to use as template in qRT-PCR. qRT-PCR was performed using a LightCycler 480 system (Roche Diagnostics) and the LightCycler 480 SYBR Green I Master kit (Roche Diagnostics, 2×), using 2 μl of cDNA, 1 μl of each primer (10 μM) in a final volume of 20 μl. Reactions were done in triplicate wells for each sample. The amplification reactions consisted of denaturation (95 °C for 10 min), amplification repeated for 40 cycles (95 °C for 10 s, 52 °C for 20 s, 72 °C for 20 s), melting curve (95 °C for 5 s, 65 °C for 1 min with continuous fluorescence measurement at 97 °C) and cooling (40 °C for 10 s). The gene encoding DNA gyrase subunit A, *gyrA*, was used as the reference gene for normalization of cDNA amount. A single amplified PCRs product was confirmed by melting curve analysis.

Results and discussion

Global gene expression profiling of *S. auresu* in the presence of azo dyes

Our previous studies demonstrated that *S. aureus* USA strain FPR3757 is capable of degrading both water-soluble Orange II and water-insoluble Sudan III azo dyes [57]. In the present study, the degradation of the azo dyes was monitored by spectrometry. We observed that 45.3 and 38.6 % of Orange II and Sudan III were reduced when the cultures were incubated for 18 h, respectively, indicating the cells were metabolically active. At this stage, the cultures were collected for total RNA isolation. In order to investigate the effects of azo dyes on gene expression patterns in *S. aureus*, whole genome microarray analysis was performed. The complete genome sequence of *S. aureus* USA300 strain FPR3757 (namely ATCC BAA 1556) has been published [18]. Genes were considered to be differentially expressed based on a change at least 1.5-fold up- or down-regulated and *p*-value less than 0.05 in comparison with controls. The microarray data revealed that water-soluble Orange II (6 μg/ml) affected the expression of 21 genes in *S. aureus* at 18 h. While water-insoluble Sudan III (6 μg/ml) affected the expression of 57 genes under the same growth conditions. The genes affected by Orange II and Sudan III were about 0.82 and 2.22 % of the total genome, respectively. The effects of Sudan III on gene expression profile of *S. aureus* appeared to be more pronounced with 51 up- and 6 down-regulated genes, compared to 8 up- and 13 down-regulated genes after the Orange II exposure (Table 3). The Venn diagram

(not shown) delineated the numbers of differentially expressed genes upon exposure to these two azo dyes. Among these differentially expressed genes, *msrA* (SAUSA300_1317, encoding methionine-S-sulfoxide reductase) was the only gene shared by exposure to both Orange II and Sudan III and it was down-regulated (Tables 4, 5). The data suggested that the effects of Orange II on *S. aureus* gene expression profile were different in compared with those of Sudan III, which is consistent with our previous observation that the mechanisms of the bacterial degradation of water-soluble and water-insoluble azo dyes might be distinct [81].

Genes differentially expressed in the presence of Orange II

There were 21 differentially expressed genes in the presence of Orange II based on the statistical analysis. All 21 genes sorted by functional COG category are shown in Table 4. Out of these, approximate half of the differentially expressed genes (47.6 %) are in the poorly characterized category or not in COGs, and 1, 5 and 5 genes involve in message storage and processing (4.8 %), cellular processes and signaling (23.8 %) and metabolism (23. 8 %), respectively. Subcellular localizations of proteins encoded by 21 differentially expressed genes were predicted, of which 10 are cytoplasmic (47.6 %), 4 cytoplasmic membrane (19.1 %), 2 cell wall (9.5 %) and 5 unknown (23.8 %, Table 4).

In message storage and processing functional category, only one differentially expressed gene (SAUSA300_0785) encoding a cytoplasmic acetyltransferase in COG K (correspond to transcription) was down-regulated 1.56-fold. This acetyltransferase belongs to Gcn5-related *N*-acetyltransferase (GNAT) superfamily, which catalyzes the transfer of an acetyl group from acetyl coenzyme A (AcCoA) to an acceptor substrate and release both CoA and the acetylated product [20, 41]. They are important for pathogenic bacteria because they contribute to emergence of drug resistance by acetylating and rendering antibiotics inactive [20, 41]. Hence, the down-regulation of SAUSA300_0785 gene may result in drug-sensitive *S. aureus* cells.

Five genes were grouped into cellular processes and signaling functional category, of which, *phoH* (encoding phosphate starvation-induced protein located in cytoplasm, SAUSA300_1531) and *agrB* (encoding accessory gene regulatory protein B located in cytoplasmic membrane, SAUSA300_1989) classified into COG T (correspond to signal transduction mechanisms) were up- and down-regulated with 1.50- and 1.75-fold, respectively. The PhoH shares 63.2 % amino acid identity with PhoH (BSU25340) in *Bacillus subtilis*, which involves in phospholipid metabolism and RNA modification [35]. The *S. aureus* quorum-sensing system is encoded by the accessory gene regulator (*agr*) locus, which consists of 5 genes (*hld*, *agrB*, *agrD*, *agrC*, and *agrA*) [8]. Agr has been described previously to be associated with reduced vancomycin susceptibility as well as virulence in *S. aureus* [56, 63, 67]. Our finding implies that the vancomycin resistance and virulence in *S. aureus* may be decreased after Orange II exposure. SAUSA300_2468 encoding an acetyltransferase with unknown subcellular location in COG M (cell wall/membrane/envelope biogenesis) was detected to be 1.75-fold down-regulated. Another two genes, SAUSA300_1534 and *msrA* in COG O (post-translational modification, protein turnover, and chaperones) also showed to be down-regulated. *msrA* encodes a methionine-S-

sulfoxide reductase, which is crucial for the protection of cellular damage from oxidative stress [6, 68, 72]. Moreover, for some bacterial pathogens, *msr* is a potential virulence determinant and the down-regulation of *msrA* could affect resistance to oxidative stress and virulence of *S. aureus* [21, 28, 68].

There were 5 differentially expressed genes involved in metabolism after Orange II exposure. Among them, 2 genes, SAUSA300_0381 and *mnhA* (SAUSA300_0855) fell into COG C (correspond to energy production and conversion). SAUSA300_0381 encoding a putative NAD(P) Hflavin oxidoreductase located in cytoplasm with 1.92-fold change in the present of Orange II. Whereas Na^+/H^+ antiporter subunit A encoded by *mnhA* was 1.64-fold down-regulated and located in cytoplasmic membrane. The putative NAD(P)H-flavin oxidoreductase (SAUSA300_0381) shares 99.6 % amino acid identity with NfrA (SA0367) in *S. aureus* (strain NCTC 8325), which has been proven to be involved in oxidative and disulfide stress responses [75]. Similarly, for *B. subtilis*, the expression of *nfrA* gene was induced upon heat shock and oxidative stress [53, 54]. NfsA (ECK0842) in *E. coli* shares 37 and 36.3 % amino acid identity with the NfrA protein of *S. aureus* NCTC 8325 and the putative NAD(P)H-flavin oxidoreductase (SAUSA300_0381), respectively. It is an oxygen-insensitive nitroreductase and acts as a lawsone-dependent azo reductase under anaerobic conditions [65]. Previous studies also have indicated that pollutant degrading bacteria might undergo the oxidative stress when exposed to pollutants [27, 29, 88]. Likewise, sulfonated azo dyes Reactive Orange 16 (RO16) and Reactive Red 120 (Red HE3B) generated oxidative stress when they were degraded by *Lysinibacillus sp*. RGS and bacterial consortium, respectively [3, 61]. Therefore, the high expression of the putative NAD(P)H-flavin oxidoreductase (SAUSA300_0381) might not only protect the cells of *S. aureus* from oxidative stress generated by sulfonated azo dyes Orange II but also has a probable role in decolorization of Orange II. *mnhA* encodes a Na^+/H^+ antiporter subunit A, which is widely distributed in cell membranes from bacteria to mammals. In *S. aureus,* the antiporter extrudes Na^+ or Li^+ in exchange for H^+ and can make the cells survive under alkaline conditions, up to pH 9.5 [33]. Therefore, it seems that the alkaline pH tolerance of *S. aureus* will be decreased due to down-regulation of *mnhA*. In COG H (correspond to coenzyme transport and metabolism), SAUSA300_1585 and *moaC* (SAUSA300_2225) were found to be 1.52-fold up- and 1.53-fold down-regulated, respectively. Both of them were predicted to be located in cytoplasm. MoaC has an amino acid sequence identity of 50.31 and 53.66 % to *E. coli* and *B. subtilis* MoaC, respectively. This gene encodes a molybdenum cofactor (Moco) biosynthesis protein C (MoaC), which is involved in the first step of Moco biosynthesis [84]. Moco is considered to be an essential component that is required by large amounts of enzymes involved in global cycles of carbon, nitrogen and sulfur [38, 52]. *acpP* (SAUSA300_1125) encodes a cytoplasmic acyl carrier protein classified into COG I or Q (corresponding to lipid transport and metabolism and secondary metabolites biosynthesis, transport and catabolism, respectively). It was detected 1.74-fold down-regulated in the presence of Orange II. Acyl carrier protein (ACP) is the carrier of fatty acids during their synthesis and utilization, which has been found to play an essential part in a growing number of processes outside of fatty acids biosynthesis [36].

It is noteworthy that SAUSA_0113 encoding immuno-globulin G-binding protein A precursor in COG R (general function prediction only) was down-regulated. The protein was

predicted to anchor cell wall and shares 98.3 % amino acid identity to SPA (staphylococcal protein A encoded by *spa*) of *S. aureus* strain NCTC 8325. Protein A from *S. aureus* has been suspected to play a role in the virulence of *S. aureus* [26, 46]. Therefore, it appears that Orange II can decrease the virulence of *S. aureus* as a pathogen. Up-regulated *asp23* in COG S (function unknown), which might play a key role in alkaline pH tolerance of *S. aureus* [42] was relevant. Therefore, our results imply that the *S. aureus* has many interlaced mechanisms through a balance between up and down regulated gene expression for adaptation to Orange II. Additionally, it was of interest to find that all 4 differentially expressed genes encoding cytoplasmic membrane proteins were down-regulated in the presence of Orange II (Table 4).

Genes differentially expressed in the presence of Sudan III

Exposure of *S. aureus* cells to Sudan III revealed 57 differentially expressed genes, which amounted for 2.22 % of the total genome (Table 3). All these genes sorted by general COGs are shown in Table 5. Among these 57 genes, there were 3, 6, and 25 differentially expressed genes involved in message storage and processing (5.3 %), cellular processes and signaling (10.5 %) and metabolism (43.9 %), respectively. Unfortunately, there are still 11 and 12 differentially expressed genes, whose functions are poorly characterized (19.3 %) or not in COGs (21.1 %). Subcellular localizations of 57 differentially expressed gene products were predicted, of which 23 are cytoplasmic, 24 cytoplasmic membrane, 1 cell wall, 1 extracellular and 8 unknown (Table 5).

In message storage and processing functional category, 3 genes involved in transcription (COG K) and DNA replication, recombination, and repair (COG L) were up-regulated (Table 5). Among them, there are 2 genes encoding transcriptional regulators, including one with similarity to the multiple antibiotic resistance regulator (MarR) family (SAUSA300_0334, 1.55-fold) and the other with glutamine synthetase repressor (SAUSA300_1200, GlnR, 1.73-fold). MarR homologs regulate activity of genes involved in antibiotic resistance, stress responses, virulence or catabolism of aromatic compounds [13, 15, 50, 60]. The up-regulation of *marR* may be used by the bacterium for detoxification in response to Sudan III. In *B. subtilis*, GlnR plays a key role in regulation by directly controlling expression of glutamine synthetase as well as several other genes involved in nitrogen metabolism [69, 70, 80]. The up-regulation of *glnR* may relieve stress of nitrogen restriction caused by Sudan III in the bacterium.

In cellular processes and signaling functional category, there are four differentially expressed genes in COG M (cell wall/membrane/envelope biogenesis, Table 5). *sgtB* (SAUSA300_1855) and *php3* (SAUSA300_1512) encoding monofunctional glycosyltransferase (MGT) and penicillin-binding protein 3 (PBP3) were up-regulated with 1.53- and 1.94-fold change, respectively. MGT has been demonstrated to play a key role in the elongation of peptidoglycan chains and PBPs also involve in the final stages of peptidoglycan biosynthesis in *S. aureus* [62, 78]. Both *sgtB* and *pbp3* might involve in cell wall biosynthesis. It has been shown that over 60 % of the dyes (Sudan II or Sudan IV) penetrated into *E. coli*, and 90 % of these penetrated dyes remained on the membrane of the bacterium [43]. The accumulation of Sudan dyes on the membrane could cause damage to

the bacterial cell walls. Therefore, the up-regulation of *sgtB* and *pbp3* may be used by the bacterium for preventing the cell wall damage mediated by Sudan III. Whereas, another gene in COG M or G (carbohydrate transport and metabolism), SAUSA300_0394 encoding cytoplasmic FAD/NAD(P)-binding Rossmann fold Superfamily protein, which may function as oxidoreducatse [39], was down-regulated 1.98-fold. *spsA* (SAUSA300_0867), encoding cell wall anchor of signal peptidase IA in COG U (intracellular trafficking, secretion and vesicular transport), showed 1.65-fold up-regulated. In *S. aureus*, it was predicted that SpsA is an inactive signal peptidase homologue and may provide some other unknown functions for the bacterium [34]. *msrA* was also down-regulated with 1.55-fold in the presence of Sudan III exposure similar to Orange II. Studies on genotoxicity of Sudan I and Sudan IV have been showed that they can induce oxidative stress in HepG2 cells [2, 87]. So similar compound Sudan III may cause oxidative stress in *S. aureus* and inhibit the expression of *msrA*.

Twenty five differentially expressed genes within metabolism functional category were all up-regulated (fold change ranging from 1.51 to 3.14). They were grouped into 7 COG categories such as C, G, E, F, I, P and Q, which correspond to energy production/conversion, carbohydrate transport/metabolism, amino acid transport/metabolism, nucleotide transport/metabolism, lipid transport/metabolism, inorganic ion transport/metabolism, secondary metabolite biosynthesis/transport/catabolism, respectively.

SAUSA300_0170 and *pckA* (SAUSA300_1731) in COG C were significantly up-regulated with 1.88- and 2.31-fold, respectively (Table 5). SAUSA300_0170 encodes an aldehyde dehydrogenase involving in the catabolism of ethanol, which is oxidized via acetaldehyde into acetate. *pckA* encodes a phosphoenolpyruvate carboxykinase (PEPCK), which catalyzes the first committed step of gluconeogenesis pathway and likely plays a key role in the growth and survival of *S. aureus* in the absence of glucose [71]. *gltT* (SAUSA300_2329) also in COG C, encoding cytoplasmic membrane-bound proton/sodium-glutamate symport protein, showed a 1.67-fold increase. GltT in *S. aureus* is homologous to GltT in *Bacillus stearothermophilus* and *Bacillus caldotenax* (55.24 % identity for both) [76]. In bacteria, glutamate transporters participate in the nutrient uptake and are crucial to viability of cells [83]. So the binding of Sudan III to *S. aureus* cells might prevent the absorption of nutrition such as glucose and glutamate resulting in the up-regulation of *pckA* and *gltT*.

Four genes in COG G were up-regulated, of which 3 genes encode membrane transporter proteins, such as SAUSA300_2449 (encoding a putative transporter), *mtlF* (SAUSA300_2105, encoding mannitol-specificphos-photransferase system, IIBC component and *glpT* (SAUSA300_0337, encoding glycerol-3-phosphate transporter) with1.53-, 1.67- and 1.90-fold increases, respectively. It has been found that *mtlF* (SA1960) in *S. aureus* was up-regulated in late growth phase related to carbon metabolism under vancomycin stress conditions and in bio-film formation, respectively [4, 9, 51]. A *glpT* mutant of *B. subtilis* has been found to be defective in uptake of glycerol-3-phosphate [44]. Our results suggest that *S. aureus* could uptake other available carbon sources (like mannitol and glycerol-3-phosphate) after some of glucose prevented to into bacterium by the binding of Sudan III on the bacterial cell. Another up-regulated gene in COG G is *zwf* (SAUSA300_1454), which encodes cytoplasmic glucose-6-phosphate 1-dehydrogenase

(G6PD) and catalyzs the oxidation of glucose 6-phosphate to gluconolactone 6-phosphate with either $NADP^+$ or NAD^+ as electron acceptor [66]. Up-regulation of *zwf* as a response to oxidative stress, recovery from heat stress, and even virulence has been demonstrated for gram-negative bacteria [30, 40, 47, 48]. The expression of *zwf* was found to be increased under oxidative stress conditions and acid-shock for *Enterococcus mundtii* CRL35 and *S. aureus* 50583, respectively [5, 66]. Similarly oxidative stress possibly generated by Sudan III could induce the up-regulation of *zwf* of *S. aureus*.

Eight genes identified as up-regulated belong to COG E. Especially, the products of SAUSA300_1231, 2538, 1808 and 0914 genes seem to function as amino acid transport. SAUSA300_1231 encodes a γ-aminobutyrate (GABA) permease, which has an amino acid sequence identity of 39.48 % to *B. subtilis* GabP protein involving not only uptakes the GABA as nitrogen source but also transports proline as a nutrient [25, 86]. It has been found that the expression of *gabP* in *B. subtilis* was induced during nitrogen-limited growth [7, 25]. An amino acid permease family protein is encoded by SAUSA300_2538, which shares 64.58 % amino acid sequence identity with BcaP in *Lactococcus lactis* [17]. BcaP has been confirmed to be the major branched-chain amino acids (BCAAs) carrier and the deletion of *bcaP* results in the loss of most of the BCAAs uptake [17]. It has been demonstrated that SAUSA300_1808 encoding an amino acid ABC transporter functions as an arogenate dehydratase, which converts L-arogenate to L-phenylalanine, and its activity is inhibited by phenylalanine (http://microcyc.genoscope.cns.fr/STAA3F1776/NEW-IMAGE?type=PATHWAY&object=PWY-3462). SAUSA300_0914 encodes a sodium/alanine symporter family protein. Three genes encoding cytoplasmic proteins in COG E involve in amino acid metabolism. Particularly, *argG* (SAUSA300_0864) encoding cytoplasmic argininosuccinate synthase and *ilvD* (SAUSA300_2006) encoding dihydroxy-acid dehydratase were observed to be significantly up-regulated (3.14 and 2.06-fold changes, respectively). These enzymes are involved in arginine biosynthesis II and BCAAs (isoleucine and valine) biosynthesis pathways, respectively. These results indicated that the binding of Sudan III to cell wall and membrane may induce amino-acids transport and biosynthesis for *S. aureus* growth.

gmk (SAUSA300_1102) was the only up-regulated gene (2.23-fold) classified into COG F. It encodes a cytoplasmic guanylate kinase involving in synthesis of nucleotide precursors and indirectly modulating the synthesis of DNA and RNA. Inhibition of Gmk would impact bacterial growth [22]. Additionally, Gmk is a potential target for novel antibacterial drugs in *S. aureus* [22]. This may indicate that excess expression of *gmk* in *S. aureus* may help the bacterium to overcome stress caused by Sudan III [57].

Four genes encoding proteins involved in lipid transport and metabolism (COG I) were up-regulated, namely *acsA* (encoding acetyl-coenzyme A synthetase, SAUSA300_1679), *accB* (encoding a biotin carboxyl carrier protein of acetyl-CoA carboxylase, SAUSA300_1476), SAUSA300_1673 (encoding 1-acyl-sn-glycerol-3-phosphate acyltransferase) and SAUSA300_0229 (putative acyl-CoA transferase FadX). Except for SAUSA300_0229 with unknown location, the other 3 gene products were predicted in cytoplasm. AcsA in *S. aureus* has a high amino acid sequence identity of 69.7 % to AcsA in *B. subtilis*. The disruption of this gene in *B. subtilis* resulted in loss of the ability to use acetate for growth and sporulation

[32]. SAUSA300_1673 is homologous to *plsC* in *B. subtilis* with 47.03 % amino acid sequence identity. PlsC depletion brought the cessation of phospholipids synthesis, but continued fatty acid synthesis in *B. subtilis* [59]. Altogether, it appears that up-regulation of fatty acid and phospholipids synthesis enzymes allowed *S. aureus* to overcome cell membrane damage caused by the binding of Sudan III.

Four genes (SAUSA300_2176, 1005, 1879 and 0619) grouped into COG P were all up-regulated (1.52–2.45 fold). Their gene products involve in inorganic ion transport rather than metabolism (two ABC transporters, one Mn^{2+}/Fe^{2+} transporter and one sodium-dependent transporter) and are anchored in cytoplasmic membrane. In bacteria, ABC transporters play roles in nutrient uptake and in secretion of toxins and antimicrobial agents [16]. They exhibit specificity for different substrates: carbohydrates, amino acids, osmoprotectants, oligopeptides, inorganic ions, bacteriocins, and DNA [1]. They further confirmed that Sudan III leads to the damage of cell membrane. SAUSA300_1899, which encodes a conserved hypothetical cytoplasmic protein involved in secondary metabolite biosynthesis, transport and catabolism (COG Q), was also found to be 1.52-fold up-regulated.

All differentially expressed genes encoding cytoplasmic membrane proteins were identified to be up-regulated in the presence of Sudan III and most genes encode transporters. Their overexpression and functional enhancement seem to be an effective direct response to the unusual membrane environment caused by Sudan III accumulation, indicating that Sudan III is more toxic to the bacterium than Orange II which agreed with our previous study [57].

Validation of microarray data and functional identification of azoreductase by qRT-PCR

For validation of microarray data and functional identification of azoreductase, the expression of the genes, *gyrA* (SAUSA300_0006), an azoreductase gene (SAUSA300_0545, *azo1*), and randomly selected 11 genes of up or down-regulated in *S. aureus* in the presence of Orange II or Sudan III were further analyzed by qRT-PCR (Table 2). The *gyrA* gene did not show any variation in expression in the presence of Orange II and Sudan III, and therefore, was used for normalization. Consistent with the microarray data, the qRT-PCR experiment confirmed that the FMN-dependent NADPH-azoreductase Azo1 is constitutively expressed, indicating its functional responsibility for azoreduction. In addition, the expression patterns of the randomly selected genes measured by qRT-PCR were consistent with the expression values obtained by microarray analysis (data not shown).

Conclusions

In summary, this global transcriptome analysis showed that Orange II and Sudan III have apparent solubility-dependent impacts on the gene expression profiling of *S. aureus*. There were 21 (8 up- and 13 down-regulated) and 57 (51 up- and 6 down-regulated) genes differentially expressed after exposure to Orange II and Sudan III, respectively. In the presence of water-soluble Orange II, most differentially expressed genes with known functions were responsive to various stress factors, such as drugs (especially vancomycin), reactive oxygen species, and alkaline shock. However, in the presence of water-insoluble Sudan III, most differentially expressed genes were found to be up-regulated, especially genes encoding cytoplasmic membrane proteins and most of them involved in cell wall or

membrane biogenesis, biosynthesis; nutrient (carbon, nitrogen, energy, inorganic ion) uptake, transport, metabolism; and stress responses. Conclusively, the azo dyes such as Orange II and Sudan III are stress-inducible xenobiotic compounds, and bacterial metabolic and stress responses depend mainly on the physicochemical properties of azo dyes and biodegradability.

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Table 1

Chemical structures and properties of azo dyes

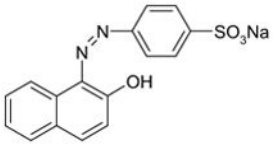
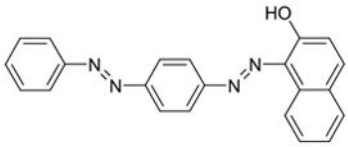
Azo dye	Chemical structure	Molecular weight	Water Solubility	Melting point (°C)
Orange II		350.32	Water-soluble (116 g/l)	164
Sudan III		352.39	Insoluble	199

Table 2

Oligonucleotide primers used in qRT-PCR

Locus tag or gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
SAUSA300_0006 (*gyrA*)	CAACCGTTGAAACAAATTGATCATC	CTGAGCGTAATGGTAATGTTGTATG
SAUSA300_1531 (*phoH*)	GGATGACAACATCGAAACTCTCT	GACGATATGAACCAATCTCAAGC
SAUSA300_0381	GGGAACCCGCAGATGAC	TTTGCGATTCCCGTTTG
SAUSA300_1200 (*glnR*)	TGCGTGGCATCTACAATCAT	TAACGGATTTAACGCCAAGG
SAUSA300_1731 (*pckA*)	GTGATTCAACCTTCGTCTTCAAC	TGCATTACCTGATTCTGAACCTT
SAUSA300_0846 (*argG*)	GTGGCGCAGCATAAGGAT	GGGCATGGAGTCGTGAAG
SAUSA300_1808	GTGGCGCAGCATAAGGAT	GGGCATGGAGTCGTGAAG
SAUSA300_0914	ACTGCGATGTATGCACAAGC	CGAACCTACGCCAGAGAAAG
SAUSA300_1679 (*acsA*)	CAGTGCAACAAACGCCTTAAT	TGGACCATTTGAGGTTGAGTC
SAUSA300_1005	GCACCCATTGTTGCACCT	CGCAAGCGATTAAATTTGC
SAUSA300_1897	TTTGATTCGTATTGGCTTCTTGT	AGTATCATCCGAATATGGCAATG
SAUSA300_0619	GGCGTCAGGTTTCGTTTTAC	CCTGGTGTTGCCCTATCATT
SAUSA300_0545 (*azo1*)	TGGCAGTGCACAAGTGAAT	CCGCCATTGCTTTCTCTTT

Table 3

Summary of differentially expressed genes in *S. aureus* incubated with Orange II and Sudan III

	Total number of genes affected[a]			
Azo dye (6 μg/ml)	**Up-regulated**	**Down-regulated**	**Total**	**% Genome**
Orange II	8	13	21	0.82
Sudan III	51	6	57	2.22

The data were from triplicate incubations

A gene was identified as significant differentially expressed if p value <0.05 and the change ≥1.5-fold (up- or down-regulated) in comparison with control

[a]The cultures were incubated for 18 h

Table 4

Genes differentially expressed in the presence of Orange II

Function and locus tag	Gene name	COG category[a]	Description of gene product	*p*-value	Putative location	Fold change
Message storage and processing						
SAUSA300_0785		K	Acetyltransferase, GNAT family	0.0414	Cytoplasmic	−1.56
Cellular processes and signaling						
SAUSA300_1531	*phoH*	T	Phosphate starvation-induced protein, PhoH	0.0211	Cytoplasmic	1.50
SAUSA300_1989	*agrB*	TK	Accessory gene regulatory protein B	0.0495	Cytoplasmic Membrane	−1.75
SAUSA300_2468		M	Acetyltransferase, GNAT family	0.0291	Unknown	−1.75
SAUSA300_1534		O	Conserved hypothetical protein	0.0328	Cytoplasmic Membrane	−1.68
SAUSA300_1317	*msrA*	O	Methionine-S-sulfoxide reductase	0.0162	Unknown	−1.71
Metabolism						
SAUSA300_0381		C	Putative NAD(P)H-flavin oxidoreductase	0.0279	Cytoplasmic	1.92
SAUSA300_0855	*mnhA*	CP	$Na(^+)/H(^+)$ antiporter subunit A	0.037	Cytoplasmic Membrane	−1.64
SAUSA300_1585		H	Conserved hypothetical protein	0.0337	Cytoplasmic	1.52
SAUSA300_2225	*moaC*	H	Molybdenum cofactor biosynthesis protein C	0.0327	Cytoplasmic	−1.53
SAUSA300_1125	*acpP*	IQ	Acyl carrier protein	0.0439	Cytoplasmic	−1.74
Poorly characterized						
SAUSA300_0748		R	Conserved hypothetical protein	0.0128	Cytoplasmic	1.59
SAUSA300_0604		R	Hydrolase, alpha/beta hydrolase fold family	0.0461	Cytoplasmic	−2.07
SAUSA300_2590		R	Conserved hypothetical protein	0.0185	Cytoplasmic	−1.75
SAUSA300_0113		R	Immunoglobulin G binding protein A precursor	0.049	Cell wall	−1.62
SAUSA300_1249		S	Conserved hypothetical protein	0.039	Cytoplasmic Membrane	−1.62
SAUSA300_2142	*asp23*	S	Alkaline shock protein 23	0.0322	Unknown	1.87
SAUSA300_2257		S	Conserved hypothetical protein	0.0306	Unknown	1.71
Not in COGs						
SAUSA300_1698		–	Conserved hypothetical protein	0.0211	Unknown	1.98
SAUSA300_1180		–	Conserved hypothetical protein	0.0174	Cytoplasmic	−1.51
SAUSA300_1985	*sdrH*	–	Serine-aspartate repeat family protein, SdrH	0.0355	Cell wall	1.84

– Not in COGs

[a]The COGs functional categories are shown as follows: message and storage and processing [includes transcription (K)], cellular processes and signaling [includes signal transduction mechanisms (T), cell wall/membrane/envelope biogenesis (M), post-translational modification, protein turnover, chaperones (O)). Metabolism (includes energy production and conversion (C), coenzyme transport and metabolism (H), lipid transport and metabolism (I), inorganic ion transport and metabolism (P), secondary metabolite biosynthesis, transport and catabolism (Q)]. Poorly characterized [includes general function prediction only (R), function unknown (S)]

Table 5

Genes differentially expressed in the presence of Sudan III

Function and locus tag	Gene name	COG category[a]	Description of gene product	*p* value	Putative location	Fold change
Message storage and processing						
SAUSA300_0334		K	Transcriptional regulator, MarR family	0.0402	Unknown	1.55
SAUSA300_1200	*glnR*	K	Glutamine synthetase repressor	0.0456	Cytoplasmic	1.73
SAUSA300_1793		L	Conserved hypothetical protein	0.0379	Cytoplasmic	1.51
Cellular processes and signaling						
SAUSA300_1855	*sgtB*	M	Monofunctional glycosyltransferase	0.0325	Cytoplasmic membrane	1.53
SAUSA300_2314		M	Conserved hypothetical protein	0.0345	Unknown	1.51
SAUSA300_1512	*pbp3*	M	Penicillin-binding protein 3	0.0195	Cytoplasmic membrane	1.94
SAUSA300_0394		MG	FAD/NAD(P)-binding Rossmann fold Superfamily	0.0185	Cytoplasmic	−1.98
SAUSA300_0867	*spsA*	U	Signal peptidase IA	0.0117	Cell wall	1.65
SAUSA300_1317	*msrA*	O	Methionine-S-sulfoxide reductase	0.0347	Unknown	−1.55
Metabolism						
SAUSA300_0170		C	Aldehyde dehydrogenase	0.0329	Cytoplasmic	1.88
SAUSA300_1731	*pckA*	C	Phosphoenolpyruvate carboxykinase (ATP)	0.0392	Cytoplasmic	2.31
SAUSA300_2329	*gltT*	C	Proton/sodium-glutamate symport protein	0.0412	Cytoplasmic membrane	1.67
SAUSA300_2449		G	Putative transporter	0.0347	Cytoplasmic membrane	1.53
SAUSA300_1454	*zwf*	G	Glucose-6-phosphate 1-dehydrogenase	0.0309	Cytoplasmic	1.52
SAUSA300_2105	*mtlF*	G	PTS system, mannitol specific IIBC component	0.0238	Cytoplasmic membrane	1.67
SAUSA300_0337	*glpT*	G	Glycerol-3-phosphate transporter	0.0458	Cytoplasmic membrane	1.90
SAUSA300_2385		E	Putative membrane protein	0.0175	Cytoplasmic membrane	2.20
SAUSA300_1231		E	Gamma-aminobutyrate permease	0.0241	Cytoplasmic membrane	1.56
SAUSA300_0864	*argG*	E	Argininosuccinate synthase	0.0075	Cytoplasmic	3.14
SAUSA300_2538		E	Amino acid permease family protein	0.0103	Cytoplasmic membrane	1.91
SAUSA300_1808		E	Amino acid ABC transporter,	0.0332	Cytoplasmic membrane	2.45
SAUSA300_1916		E	Aminotransferase	0.0224	Cytoplasmic	1.51
SAUSA300_0914		E	Sodium:alanine symporter family protein	0.0058	Cytoplasmic membrane	2.33
SAUSA300_2006	*ilvD*	EG	Dihydroxy-acid dehydratase	0.0263	Cytoplasmic	2.06
SAUSA300_1102	*gmk*	F	Guanylate kinase	0.0317	Cytoplasmic	2.23

Function and locus tag	Gene name	COG category[a]	Description of gene product	*p* value	Putative location	Fold change
SAUSA300_1476	*accB*	I	Acetyl-CoA carboxylase, biotin carboxyl carrier	0.0277	Cytoplasmic	2.20
SAUSA300_0229		I	Putative acyl-CoA transferase FadX	0.0003	Unknown	2.17
SAUSA300_1679	*acsA*	I	Acetyl-coenzyme A synthetase	0.0072	Cytoplasmic	2.18
SAUSA300_1673		I	1-acyl-sn-glycerol-3-phosphate acyl-transferases	0.0334	Cytoplasmic	1.51
SAUSA300_2176		P	ABC transporter, ATP-binding protein	0.0267	Cytoplasmic membrane	1.52
SAUSA300_1005		P	Mn^{2+}/Fe^{2+} transporter, NRAMP family	0.0338	Cytoplasmic membrane	1.67
SAUSA300_1897		P	Sodium-dependent transporter	0.0399	Cytoplasmic membrane	2.16
SAUSA300_0619		P	ABC transporter, permease protein	0.0416	Cytoplasmic membrane	2.45
SAUSA300_1899		Q	Conserved hypothetical protein	0.0246	Cytoplasmic	1.52
Poorly characterized						
SAUSA300_1700		R	Polysaccharide biosynthesis protein	0.0085	Cytoplasmic membrane	1.77
SAUSA300_2268		R	Sodium/bile acid symporter family	0.0351	Cytoplasmic membrane	1.61
SAUSA300_1229		R	Hydrolase, haloacid dehalogenase-like family	0.0439	Cytoplasmic	1.53
SAUSA300_1685		R	Conserved hypothetical protein	0.0459	Cytoplasmic	−1.55
SAUSA300_1364	*engA*	R	GTP-binding protein EngA	0.0126	Cytoplasmic membrane	2.05
SAUSA300_0013		S	Putative membrane protein	0	Cytoplasmic membrane	1.62
SAUSA300_0733		S	degV family protein	0.047	Cytoplasmic	1.67
SAUSA300_2418		S	Conserved hypothetical protein	0.0019	Cytoplasmic	−1.53
SAUSA300_0330		S	Putative transport protein SgaT	0.0309	Cytoplasmic membrane	1.84
SAUSA300_2031		S	Conserved hypothetical protein	0.0086	Cytoplasmic membrane	1.59
SAUSA300_1284		S	Conserved hypothetical protein	0.0136	Cytoplasmic	1.59
Not in COGs						
SAUSA300_pUSA010001		–	Replication protein	0.0346	Cytoplasmic	1.95
SAUSA300_pUSA030001	*repA*	–	Replication initiator protein	0.0459	Cytoplasmic	1.87
SAUSA300_pUSA030007	*ermC*	–	rRNA adenine N-6-methyltransferase	0.0234	Cytoplasmic	2.02
SAUSA300_0661		–	Conserved hypothetical protein	0.0107	Cytoplasmic membrane	1.67
SAUSA300_0642		–	Conserved hypothetical protein	0.0334	Cytoplasmic membrane	1.58
SAUSA300_0681		–	Conserved hypothetical protein	0.0182	Unknown	1.61
SAUSA300_1011		–	Conserved hypothetical protein	0.034	Unknown	1.64
SAUSA300_2355		–	Putative lipoprotein	0.0158	Unknown	2.09
SAUSA300_1680	*acuA*	–	Acetoin utilization protein AcuA	0.0027	Cytoplasmic	2.13

Function and locus tag	Gene name	COG category[a]	Description of gene product	*p* value	Putative location	Fold change
SAUSA300_1461		–	Conserved hypothetical protein	0.0167	Cytoplasmic membrane	1.98
SAUSA300_1053		–	Conserved hypothetical protein	0.0061	Unknown	−1.81
SAUSA300_1055	*efb*	–	Fibrinogen-binding protein	0.0211	Extracellular	−1.58

– Not in COGs

[a]The COGs functional categories are shown as follows: message and storage and processing [includes transcription (K), replication, recombination and repair (L)]. Cellular processes and signaling [includes cell wall/membrane/envelope biogenesis (M), intracellular trafficking, secretion and vesicular transport (U), post-translational modification, protein turnover, chaperones (O)]. Metabolism [includes energy production and conversion (C), carbohydrate transport and metabolism (G), amino acid transport and metabolism (E), nucleotide transport and metabolism (F), lipid transport and metabolism (I), inorganic ion transport and metabolism (P), secondary metabolites biosynthesis, transport and catabolism (Q)]. Poorly characterized (includes general function prediction only (R), function unknown (S))