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Citation: Appl. Phys. Lett. **106**, 213701 (2015); doi: 10.1063/1.4921754
View online: http://dx.doi.org/10.1063/1.4921754
View Table of Contents: http://aip.scitation.org/toc/apl/106/21
Published by the American Institute of Physics

Genetic effects of an air discharge plasma on *Staphylococcus aureus* at the gene transcription level

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(Received 1 December 2014; accepted 15 May 2015; published online 26 May 2015)

The dynamics of gene expression regulation (at transcription level) in *Staphylococcus aureus* after different doses of atmospheric-pressure room-temperature air plasma treatments are investigated by monitoring the quantitative real-time polymerase chain reaction. The plasma treatment influences the transcription of genes which are associated with several important bio-molecular processes related to the environmental stress resistance of the bacteria, including oxidative stress response, biofilm formation, antibiotics resistance, and DNA damage protection/repair. The reactive species generated by the plasma discharge in the gas phase and/or induced in the liquid phase may account for these gene expression changes. © *2015 AIP Publishing LLC*.
[http://dx.doi.org/10.1063/1.4921754]

The inactivation effects of many types of atmospheric-pressure non-thermal plasmas on various kinds of bacteria living under different conditions as well as underlying mechanisms have been investigated in order to identify new bacteria-killing methods to reduce the use of antibiotics.[1–8] Plasma generated and/or induced reactive species are considered to play pivotal roles in aseptic processes because they cause functional perturbances and structural damages in bacteria.[9–12] However, for those bacteria which still possess intact genome structures and functions during plasma exposure, it is possible that the expression of many genes may adapt to influence the related intracellular bio-molecular processes in order to protect themselves and self-repair. Consequently, the intricate bio-macromolecular regulatory mechanism in *S. aureus* upon plasma exposure at the gene expression level is crucial to a better understanding of the physiological regulation, plasma inactivation mechanism, and possible bacteria-plasma resistance in order to optimize plasma medicine.

In this work, gene transcription analysis is performed by quantitative real-time polymerase chain reaction (q-rt-PCR) to investigate how *S. aureus* responds to different doses of plasma exposure and subsequent gene expression changes. A home-made atmospheric-pressure air plasma source (Fig. 1) is utilized to generate plasma on *S. aureus* suspension, because in bio-medical applications, plasma discharges are often generated between electrodes in the apparatus and the exudative tissue fluids with bacteria attached on and/or near the wounds on human bodies. The air plasma is driven by a 10 kV DC voltage, and an array with 105 fine copper rods (radius = 1 mm) is used as the electrode. The discharge current is about 5 mA being restricted by the ballast resistors R_1 (25 MΩ) and R_2 (25 MΩ). Consequently, the gas temperature of the plasma is within the range of 290–300 K and can be touched safely by human body.

The *S. aureus* NCTC 8325 cells were incubated in fresh sterile brain heart infusion (BHI) medium overnight to approximately 1–2 × 10^9 CFU/ml, and plasma was generated on 4 ml of the bacterial suspension in a petri dish. Distance between the tips of multi-electrodes and upper surface of the bacterial suspension was fixed at 10 mm. The suspension

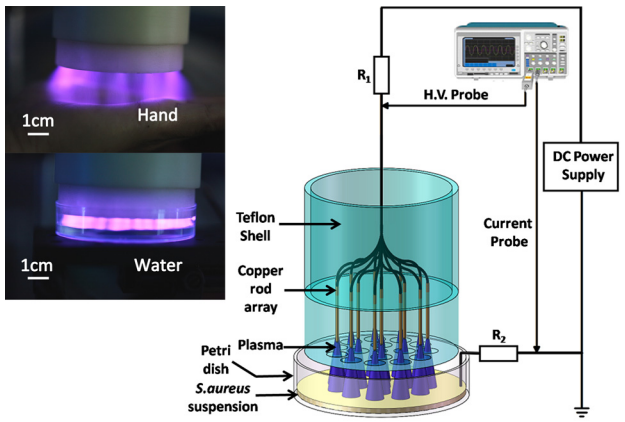

FIG. 1. Schematic of the atmospheric-pressure room-temperature air plasma source and photographs of the air plasma.

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was manually stirred by pipetting up and down per 5 min during the treatment process. After plasma exposure, the total cellular RNA of the *S. aureus* was extracted immediately.[13] In the reverse transcription, the cDNAs were synthesized using a PrimeScript 1st Strand cDNA Synthesis Kit. The oligonucleotide primers of four groups of representative genes involved in several important cellular responses upon environmental stress in *S. aureus*, including oxidative stress response, biofilm formation, antibiotics resistance, and DNA damage protection/repair, were designed by Vector NTI Suite software using sequences from the *S. aureus* DNA database maintained by National Center for Biotechnology Information (NCBI).[14,15]

Q-rt-PCR was performed with SYBR Premix Ex Taq™ on the StepOne Real-Time PCR System. The reaction mixture was initially incubated for 3 min at 95 °C, followed by 40 cycles for 10 s at 95 °C, 30 s at 58.9 °C, and 20 s at 72 °C. The relative quantification based on the expression abundance of a target gene versus the housekeeping gene 16SrRNA considered to be an endogenous control was calculated by Stratagene Mx3000P qPCR software to determine the gene expression changes on the transcription level. The standard and melting curves were analyzed to evaluate the PCR efficiency and specificity. The P values in the Student's t-tests were calculated to determine the validity of the gene expression changes obtained from q-rt-PCR. A gene with a P value in the t-test with less than 0.05 and an absolute change in the transcript level greater than (or equal to) 2 folds was considered to be statistically significant.[16] Three independent sets of experiments were performed in triplicate for each target gene.

Group I consists a set of genes belonging to the functional class "oxidative stress response" including SodA, KatE, AhpC, Nfo, and G6PD. The expression products of these genes have respective roles in the response of the cellular antioxidant defense and are able to act collectively to avoid bacterial oxidative damage.[17,18] As shown in Fig. 2, the expressions are all up-regulated after plasma treatment for 10 and 30 min. The results indicate a series of anti-oxidative reactions occurring in *S. aureus* after plasma exposure. Specifically, *S. aureus* has a kind of superoxide dismutase (SOD) encoded by SodA and behaves as the major dismutase

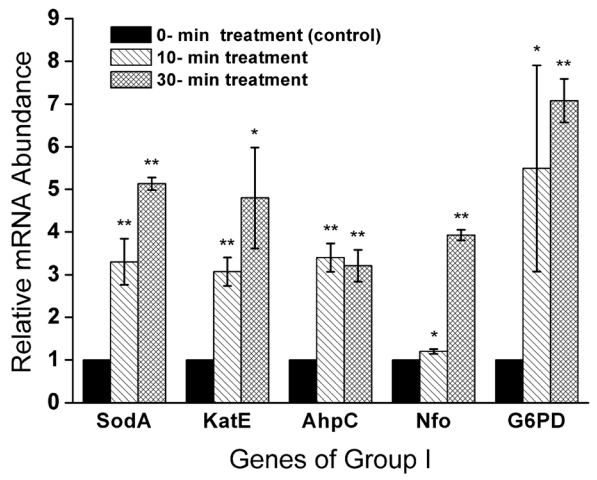

FIG. 2. Relative expression changes of the *S. aureus* genes related to oxidative stress response after plasma exposure for 10 and 30 min.

for scavenging internally-generated O_2^- produced from bacterial aerobic metabolism, which expression can be strongly up-regulated by the enhanced concentration of internally-generated O_2^- in *S. aureus*.[19,20] The fact that the SodA expressions increase by 3.30 and 5.13 folds suggests that the plasma treatment can disrupt the equilibrium of intracellular superoxide production in the bacteria. Moreover, the expressions of KatE coded for catalase (H_2O_2 scavenger) change by 3.07 and 4.80 folds to counteract the greatly enhanced intracellular H_2O_2 concentrations after the plasma treatment. In the meantime, the expressions of alkyl hydroperoxide reductase encoded by AhpC are stimulated by 3.40 and 3.21 folds. This enzyme plays a protective role in the bacteria through its peroxidase activity by reducing hydrogen peroxide, peroxynitrite, and organic hydroperoxides,[21] thus showing that these oxidants in the plasma-treated bacteria exceed the normal amounts. In addition, the increasing expressions of Nfo (encodes for endonuclease IV, a DNA repair protein) by 1.2 and 3.92 folds imply that the bacterial DNA structures are gradually damaged with the increase in plasma exposure. The induced expressions of G6PD (codes for glucose-6-phosphate dehydrogenase, an enzyme for reconstitution of the NADPH pool) by 5.49 and 7.08 folds suggest large consumption of reductive NADPH in the bacteria to combat the excessive intracellular oxidative species.

S. aureus shows a sophisticated bio-molecular mechanism to detect intracellular oxidant concentrations and protects itself from oxidative stress by activating the antioxidant defense genes.[17,18] Fig. 2 shows that the plasma treatment induces oxidative stress in the bacteria as the concentrations of the aforementioned intracellular reactive oxygen species (ROS) exceed the normal doses and the subsequent oxidative stress response may be among the most important bio-chemical mechanisms for *S. aureus* to offset the deleterious effects caused by the plasma treatment. Besides, environmental factors such as ionization, UV radiation, as well as redox-cycling agents can trigger the bacterial intracellular oxidative stress response. Here, the expression changes of the related genes (Group I) in *S. aureus* upon plasma exposure are similar to the treatment effects of some pro-oxidants such as mupirocin and mitomycin C.[22] It is, however, unclear whether *S. aureus* elicits similar response and/or develops novel strategies to cope with the induced intracellular oxidative stress between the plasma treatment and other irritants.

Biofilms can be initiated by planktonic *S. aureus* on human skin wounds and are responsible for many undesirable effects such as chronic wounds and nosocomial infections. Group II focuses on some specific genes that are responsible for the complex pathways involved in the regulation of *S. aureus* biofilm formation. According to Fig. 3, the expressions of Group II genes are all up-induced after plasma exposure. The expression of IcaA, which is responsible for the polysaccharide intercellular adhesion (PIA) synthesis as a key factor for the planktonic *staphylococci* to attach, aggregate, and form hydrated extracellular polymeric substances (EPS), are considerably stimulated by 4.23 and 1.90 folds. SarA is an essential element in the synthesis of PIA and biofilm development in *S. aureus*, and the expressions are up-regulated by 4.57 and 4.25 folds. SigB, especially important in the stability of *Staphylococcal* biofilms

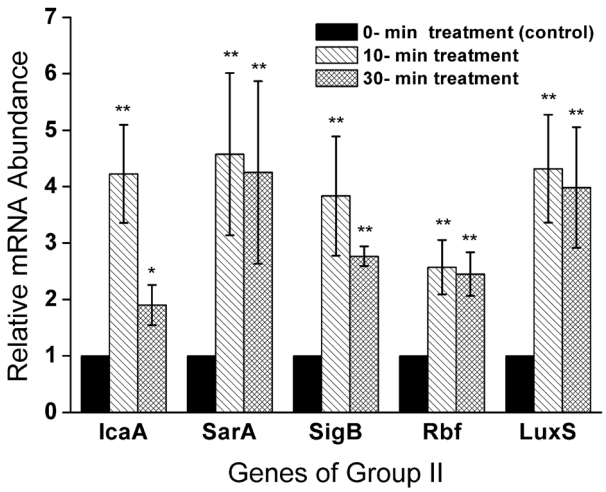

FIG. 3. Relative expression changes in the *S. aureus* genes related to biofilm formation after plasma exposure for 10 and 30 min.

under environmental stress, is up-induced by 3.84 and 2.77 folds, respectively. Rbf, which encodes the AraC family transcriptional regulator and promotes biofilm formation by *S. aureus*, shows enhanced expression by 2.57 and 2.45 folds. In addition, LuxS, whose expression results in the formation of autoinducer-II and links to the inhibition of production of PIA in *staphylococci*, also shows statistically significant increases of 4.32 and 3.98 folds.[23] Consequently, *S. aureus* responds to the plasma-driven stress to regulate biofilm formation *via* bio-molecular processes on the genetic level. However, LuxS is a down-regulator of PIA production, whereas all others are promoters. Therefore, it is not easy to simply infer whether the plasma treatment facilitates or attenuates the biofilm formation. As reported previously,[16,24] H_2O_2 or acid treatment of *S. aureus* in suspensions suppresses the transcription of IcaA, thereby proposing the possibility that apart from H_2O_2 or H^+, other plasma generated and/or induced reactive species in the plasma-treated bacterial suspension have stronger up-regulating impact on this gene.

Figure 4 displays the plasma treatment effects on the expression of several antibiotics resistance related genes in *S. aureus*. The expressions of the methicillin resistance factor (encoded by FemA) are up-regulated by 3.12 and 3.09 times. A putative drug efflux pump protein (encoded by EmrB/QacA) is stimulated and enhanced by 3.02 times after plasma exposure for 10 min, while it shows an insignificant change of 1.56 folds after exposure for 30 min, similar to MrpF/PhaF, which codes the multiple resistance and pH regulation family protein F by 2.39 and 1.19 folds. However, NorA, which codes another putative drug efflux pump protein (multidrug major facilitator superfamily transporter), exhibits no significant changes after exposure for either 10 or 30 min (1.67 and 1.17 folds). Moreover, 10-min plasma exposure does not induce significant gene expression changes in the fosfomycin resistance protein (encoded by FosB) of 1.17 folds and even represses its mRNA abundance to 0.59 folds after the 30-min treatment. Hence, it is suggested that *S. aureus* may show potential changes in its resistance to different antibiotics after different doses of plasma treatment due to the gene sensitivity to the plasma-driven environmental alteration.

Plasma treatment of cells can cause structural and functional damage in DNA.[25,26] According to Fig. 5, for the DNA-damage protection/repair-related genes in Group IV, the expression of RecG which encodes an ATP-dependent DNA helicase increases by 4.18 and 3.23 folds after plasma treatment for 10 and 30 min. Besides, the expression of RadA, which codes a DNA repair protein, is up-regulated by 4.51 and 4.28 times, and that of RecN, which codes another DNA repair protein, goes up by 2.17 and 2.44 times. The expression of Dps, which encodes a DNA-binding ferritin-like family protein and plays a central role in protecting the DNA from oxidative damage by directly binding to DNA, is enhanced by 2.87 and 3.13 folds. Specifically, Nfo in Group I is also responsible for DNA damage repair which shows a 3.92 folds increase after exposure for 30 min. Therefore, a regulatory network involved in many DNA damage protection/repair-related genes and pathways is activated. The results indirectly prove that plasma exposure damages the DNA structure and perturbs its function in *S. aureus*, and are in good agreement with the DNA content analyses by utilizing agarose gel electrophoresis and fluorescence activated

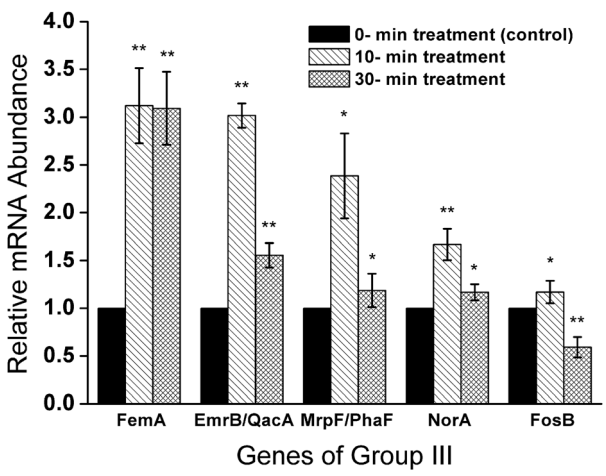

FIG. 4. Relative expression changes in the *S. aureus* genes related to antibiotics resistance after plasma exposure for 10 and 30 min.

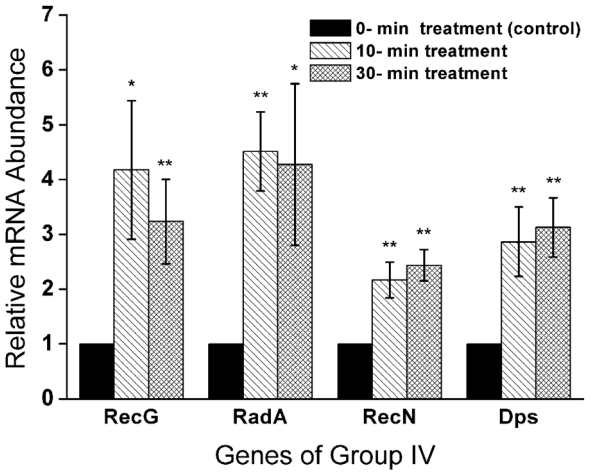

FIG. 5. Relative expression changes in the *S. aureus* genes related to DNA damage protection/repair after plasma exposure for 10 and 30 min.

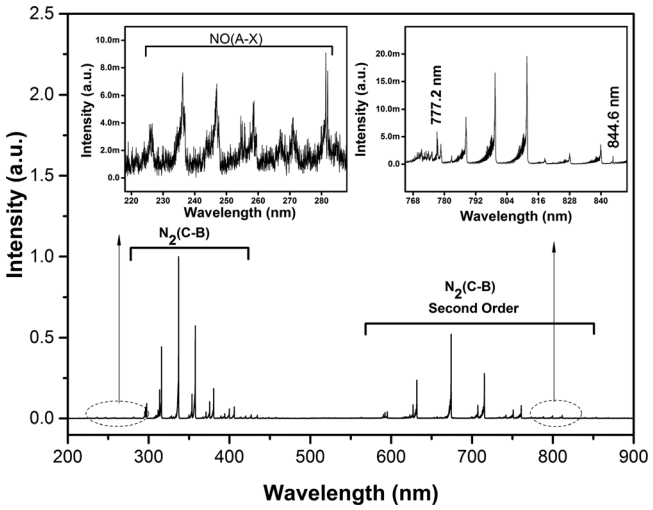

FIG. 6. Optical emission spectrum (OES) of the air plasma acquired at 10 mm below the electrode array.

cell sorting (FACS) technique on the plasma-treated cells, virus, and naked DNA.[25–27]

Figure 6 exhibits the typical emission spectrum of this air plasma (200–900 nm). The most intense emissions are observed between 300–450 nm and 600–800 nm, which are attributed to N_2(C-B) and the second order emission from N_2(C-B).[28] The emission bands of the NO_γ-system at 200–300 nm (NO(A-X)) as well as reactive atomic oxygen at 777.2 nm and 844.6 nm are also detected. The plasma discharges at the gas-phase and gas–liquid interface can initiate a large number of plasma-chemical reactions in various types and then form large amounts of primary and secondary reactive species in the gas and gas–liquid phases. Thereafter, the reactive species can dissolve and penetrate the liquid impacting the bacteria in the suspension *via* a series of biochemical reactions.[29] The reactive species generated from the plasma, for example, charged particles, reactive atomic oxygen, NO, and OH•, lead to the ultimate generation of ROS/RNS (H_2O_2, NO_2^-, NO_3^-, O_3, etc.) in the suspension and associated pH value decrease.[30] Multiple kinds of ROS/RNS can effectively influence the bacterial gene expressions, acting as secondary messengers responsible for signal transduction.[31,32] Therefore, the plasma-driven gene expression regulations in *S. aureus* may result from the combined action of the reactive species generated and/or induced in the gas, gas-liquid, and liquid phases, but better understanding of their relative contributions requires more work.

In this work, the cellular response of *S. aureus* to different doses of air plasma treatment is analyzed at the gene transcription level. The results show that 10- and 30-min plasma exposure can induce gene expression changes to a different degree. It involves several important bio-molecular regulatory processes, including oxidative stress response, biofilm formation, antibiotics resistance, and DNA damage protection/repair. The plasma treatment activates the oxidative stress response in *S. aureus*, changes the resistance to different antibiotics, and stimulates the intracellular DNA damage protection/repair processes to counteract. However, whether plasma exposure facilitates or attenuates the biofilm formation requires more in-depth investigation. Gene expression changes in *S. aureus* may be related to the plasma generated and/or induced reactive species.

This work was supported by the National Natural Science Foundation of China under Grant Nos. 11035005, 11005126, and 50876101, Hefei Institutes of Physical Science, Chinese Academy of Sciences (CASHIPS) Dean Funds No. YZJJ201331, City University of Hong Kong Strategic Research Grant (SRG) No. 7004188, as well as Hong Kong Research Grants Council (RGC) General Research Funds (GRF) No. CityU 112212.

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