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Original

Influence of Gas Temperature in Atmospheric Non-Equilibrium Plasma on Bactericidal Effect

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In this study, the relationship between plasma gas temperature and the bactericidal effects on five of bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Bacillus cereus* (spore)) in liquid was investigated using a temperature-controllable plasma source. We determined that the bactericidal ability improved as the plasma gas temperature increased. Specifically, the bactericidal ability on *E. coli* of 80-°C plasma was enhanced by as much as 6.3 times compared to that of 10-°C plasma. The relationship between plasma gas temperature and the amount of hydroxyl radical, singlet oxygen, hydrogen peroxide, and ozone introduced into the solution was investigated. Our results also showed that each reactive species production increased by 2.1, 9.0, 1.6, and 17 times, respectively, with 80-°C compared to 10-°C plasma. The relationship between the bactericidal ability and amount of reactive species indicated that singlet oxygen and ozone introduced to the solution mostly influenced the bactericidal ability as the plasma gas temperature increased. We conclude that the plasma gas temperature is the crucial parameter for plasma sterilization.

Key words : Temperature controllable plasma / Atmospheric low temperature plasma / Bacterial inactivation / Reactive species.

INTRODUCTION

Conventional atmospheric thermal plasma is generated by applying high voltage, and discharge damage is caused to the treated targets. Additionally, the gas temperature of several thousand degrees causes heat damage. On the other hands, atmospheric non-equilibrium plasma has attracted attention as a new sterilization device for heat sensitive objects such as fibers, polymers, and living bodies because it has a wide antibacterial spectrum at low temperatures and shows residual toxicity unlike ethylene oxide gas (Fridman et al., 2008; Graves, 2012; Takamatsu et al., 2013; Yanagawa et al., 2017). In particular, a growing number

of reports have shown evidence of bacterial inactivation on living bodies by direct plasma irradiation (O'Connor et al., 2014; Traba et al., 2013; van Gils et al., 2013). This is because plasma sources that are free from discharge damage to an object have been developed (Okazaki et al., 1993). To irradiate living bodies with plasma, accurate temperature control is required to prevent thermal damage to the target. For example, to irradiate skin with plasma, the plasma gas temperature should be controlled such that it is below the denaturation temperature of proteins (Tümmel et al., 2007). The gas temperature of general atmospheric non-equilibrium plasma is lower than that of atmospheric equilibrium plasma, which is usually several thousand degrees (Iwai et al., 2013; Kaburaki et al., 2013). However, because plasma is generated from gas at room temperature by discharge, the gas temperature of the plasma

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always becomes higher than room temperature, and many plasma sources generate plasma such that the gas temperatures are higher than the denaturation temperature of protein (Hofmann et al., 2011). In these conventional plasma sources, in order to reduce the plasma gas temperature, limiting discharge power or increasing the gas flow rate are common (Laroussi, 2005; 2010). However, in these methods, because the plasma density decreases, the treatment effect also decreases (Machala et al., 2009). For this reason, generating high power plasmas at low gas temperatures is difficult. In addition, the influence of plasma gas temperature on the bactericidal effect was not investigated because the plasma gas temperature depends on the discharge power or the gas flow rate.

To overcome these problems, we proposed a temperature-controllable plasma technique (PAT Japan: 4611409, U.S.: 8,866,389) and developed a plasma source (Oshita et al., 2015). This device can control the plasma gas temperature to between -50 and 160°C with a standard deviation of 1°C independently from the discharge power and plasma gas flow rate. In this study, the influence of the plasma gas temperature on the inactivation effect for various kinds of bacteria was investigated using the temperature-controllable plasma source without changing the discharge power and gas flow rate.

MATERIALS AND METHODS

Temperature-controllable plasma source

In our temperature-controllable plasma source, the temperature of helium gas was first adjusted through a temperature control unit. This control unit comprised a gas heater and gas cooler containing a condenser and liquid nitrogen. Helium gas was then introduced into a plasma generating unit, and plasma was generated. The temperature of the plasma gas was relayed back to the input power of the gas heater so that it could be set to a desired value.

The plasma gas temperature was measured using a thermocouple to simplify the measurement (Oshita et al. 2015). In our experiments, 3% oxygen gas was added to the temperature-controlled helium gas before being introduced into the plasma generating unit. A dielectric barrier discharge (DBD) plasma jet with two ring-shaped copper electrodes placed on the outside of a glass tube (3 mm ID, 5 mm OD) was used as the plasma generating unit. Finally, 9 kV of electric power was applied at 16 kHz to generate the plasma.

Bactericidal experiment

To evaluate the bactericidal effect of the plasma at each gas temperature, the numbers of surviving

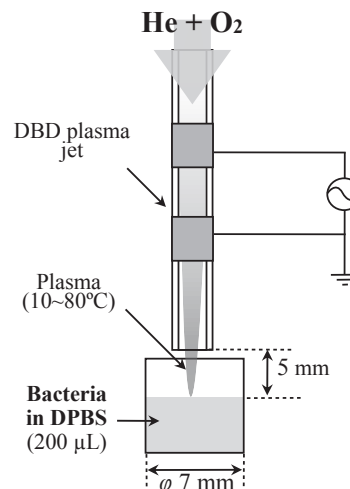


FIG. 1. Setup of the bactericidal experiment with temperature controlled plasma. Bacteria in DPBS was irradiated with temperature-controlled plasma in 2 min. The plasma was generated from 3% oxygen mixed helium gas using DBD plasma jet. The distance between the outlet of plasma and surface of the solution was 5 mm.

bacteria after plasma irradiation were investigated. *Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC27853, *Staphylococcus aureus* ATCC25923, *Enterococcus faecalis* ATCC29212, and *Bacillus cereus* JCM2152 were used as indicator bacteria ($n = 3$). *E. coli* and *P. aeruginosa* are classified a Gram-negative bacterium, which has outer membrane. *S. aureus*, *E. faecalis* and *B. cereus* are classified a Gram-positive bacterium which have structureless of outer membrane. *B. cereus* was cultured at 37°C for 7 d, and formed spore by heating at 65°C for 30 min (Sakagami et al., 1998). The bacteria were suspended in Dulbecco's phosphate-buffered saline (DPBS) (-) ($\text{pH} = 7.2-7.4$) because the bactericidal activity using plasma irradiation is affected by the pH value of the solution (Ikawa et al., 2010; Sakagami et al., 1998). The temperature of the bacterial suspension before irradiation with plasma was 23°C .

Temperature-controlled plasma was irradiated to bacterial suspension of 200 μL above the solution, and the distance between the outlet of the plasma jet and surface of the solution was 5 mm (Fig.1). The gas flow rate of helium was 9.7 standard liter per minute (SLM), whereas oxygen was 0.3 SLM. The plasma gas temperature was controlled from 10 to 80°C at an interval of 10°C . Bacterial inactivation effect by plasma treatment is considered to be caused by oxidant stress of reactive oxygen species (ROS) (Graves, 2012). There are ROS introduced into the liquid which has a lifetime as long as several microseconds and several tens of minutes in liquid, the reaction (contact) time influences the bacte-

ricidal effect. The short lifetime ROSs, which the lifetime is several microseconds, is generated in the plasma and immediately consumed by reaction with substance in the solution or bacteria. However, there is a possibility to exist ROSs with a long lifetime over several minutes which is generated by the reaction of short lifetime ROS with water molecule and the components in DPBS (Bruggeman and Leys, 2009). Thus, the influence of the reaction time of ROS and the bactericidal effect on bacteria is able to be eliminated by keeping the constant reaction time. In this study, the reaction was stopped by inactivation of ROS. To inactivate ROS, the bacterial suspension after plasma irradiation was allowed to stand at room temperature for 20 min and then mixed with LB medium at 1:1. Therefore, all of the reaction time of ROS and bacteria is all 20 min. The bacterial suspension mixed with the LB medium was dropped onto the LB agar medium and incubated at 37°C for 18 h. The number of surviving bacteria was determined using a colony count method (Takamatsu et al., 2015). In these experiments, detection limits were 200 colony forming unit per sample (CFU/sample) in all bacteria. The solution temperature after plasma irradiation for 1 min was measured, since the temperature of plasma treated solution became an equilibrium state in about 10 s at any gas temperature and the temperature was stable thereafter.

As described above, the ROS in the liquid affects the bactericidal effect of plasma. Therefore, by selectively quenching specific ROS, it is possible to investigate the involvement of specific ROS in the bactericidal effect. In this study, to investigate the involvement of singlet oxygen ($^1\text{O}_2$) in the bactericidal effect, a bactericidal experiment using L-histidine as a quencher of $^1\text{O}_2$ was carried out (Matsumura et al., 2013). The bacterial suspension of *E. coli* containing 100 mM of L-histidine was irradiated 40-°C plasma for 2/3 min.

When the lifetime of a bactericidal factor is long, amount of bactericidal factor is represented by a product of the amount of introduced bactericidal factor by plasma irradiation per unit time a mol/(min·L) and plasma irradiation time t min. The decreasing number of surviving bacteria is described using following equation.

$$\text{Log}_{10}\left(\frac{N_0}{N}\right) = kat' t \quad (1)$$

The t' is the reaction time of bacteria with a bactericidal factor, N_0 is the initial number of bacteria and N is the number of surviving bacteria after plasma irradiation for t min, k L/(mol·min) is a reaction constant. The t' is 20 min under all conditions, a and k are constant under conditions of the same plasma gas temperature and the same kind of bacteria. Therefore, it is considered that the values kat' varies according to the kind of bacteria

and the gas temperature of plasma. To compare quantitatively between the bactericidal effect of each plasma gas temperature and the amount of introduced ROS into the solution, the value of kat' is defined as the bactericidal activity BA , and the values were estimated for each kind of bacteria and gas temperatures. The value of BA was represented as the number of digits of decreased number of bacteria by plasma irradiation per unit time.

To inactivate bacteria by plasma irradiation, the necessity of plasma irradiation for over certain time like threshold has been reported (Lee et al., 2009). Therefore, in the graph of relationship between plasma irradiation time and surviving number of bacteria, a survival straight line was created from the point immediately before the point where the number of surviving bacteria became the threshold value or less. And the slope of the line was taken as the BA value. In this paper, the threshold value was set to 1/10 of the initial number of bacteria. Hence, the BA value was calculated as $BA = \log(N_1 / N_2) / (t_2 - t_1)$. Where, N_1 and N_2 are the number of surviving bacteria after plasma irradiation for t_1 and t_2 min, respectively. When the change of number of bacteria between untreated and treated for 2 min was within 1-order of magnitude, BA_1 was set to '0'. On the other hand, when the number of surviving bacteria was lower than the detection limit value within 1/3 min of the shortest irradiation time, BA_2 was set to $\log(N_0 / 200) / (1/3)$. The BA_1 in which the highest temperature point where the number of surviving number became less than the detection limit and the BA_2 in which the lowest temperature point were used, respectively.

Measurement of the generated ROS

To investigate the plasma gas temperature dependence on the amount of generated ROS, the concentration of ROS in the solution following plasma irradiation were measured. In this experiment, hydroxyl radicals ($\text{HO}\cdot$), $^1\text{O}_2$, ozone (O_3), and hydrogen peroxide (H_2O_2) in the solution were measured as ROS. The lifetime of $\text{HO}\cdot$ and $^1\text{O}_2$ are nanoseconds – microseconds and that of O_3 is tens of minutes in liquid at room temperature (Bocci, 2005; Rodgers and Snowden, 1982; Yamatake et al., 2006). The H_2O_2 is stable in diluted water at room temperature (Hayashi and Asada, 1977). However, this lifetime is shorten to several milliseconds by reactions of H_2O_2 with components in the solution such as in cell (Redmond and Kochevar, 2006). The plasma gas temperature was controlled under the same conditions as in the bacterial inactivation experiments.

The concentration of $\text{HO}\cdot$ and $^1\text{O}_2$ were measured by electron spin resonance (ESR) using spin-trapping agents (Takamatsu et al., 2015). A magnetic reso-

nance spectrum was obtained by ESR spectroscopy (JES-FA100, JEOL Ltd., Tokyo, Japan). As the spin-trapping agents of HO·, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) was used, and for $^1\text{O}_2$, 2,2,5,5-tetramethyl-3-pyrroline-3-carboxamide (TPC) was used (Takamatsu et al., 2014). These reagents were dissolved in a DPBS (-) solution, and the concentrations of DMPO and TPC were fixed at 200 and 75 mM, respectively. The ESR was set at a 9.424818-GHz microwave frequency, 100-kHz modulation frequency, 2-min sweep time, 335.5 ± 5 mT magnetic field, 0.7-mT modulation width, and 0.1-s time constant. Each solution that dissolved the individual reagent was irradiated with plasma. Because the amount of DMPO-OH, which is a DMPO spin adduct of HO·, decreases over time due to the reactivity of HO·, the time of plasma irradiation was 0.5 min according to reference (Takamatsu et al., 2014). Additionally, all introduced ROS into solution were trapped by enough concentration of each spin trapping reagent. Therefore, the concentration of HO· and $^1\text{O}_2$ increased in proportion to the plasma irradiation time, the bactericidal factor is examined using the amount of the introduced ROS per unit time which is calculated from results of ROS measurement with 0.5 min plasma irradiation.

The concentration of O_3 and H_2O_2 were measured by absorption spectrophotometry using a double-beam spectrophotometer [U-2900, Hitachi High-Technologies Co.] (Takamatsu et al., 2014). In this method, the concentration of each ROS was calculated from the absorbance of the reagents. The concentration of O_3 was measured from the absorbance of a 350-nm wavelength that decreased by reaction of O_3 with indigo reagent (Ozone AccuVac® Ampules, MR, pk/25, Hach Company (USA)). The concentration of H_2O_2 was measured from the absorbance of a 440-nm wavelength that increased by reaction of H_2O_2 with the solution that included xylenol orange 200 μM , ammonium iron (II) sulfate 150 mM, sulfuric acid 150 mM, and sorbitol 200 mM (Ikai et al., 2013). In the measurement of O_3 and H_2O_2 , 100 μL pure water irradiated plasma, and the reagent for H_2O_2 or O_3 were dissolved after plasma irradiation to estimate the influence of short lifetime ROS (e.g., HO· and $^1\text{O}_2$).

Assuming that the ROS measured in this study influenced the bactericidal effect, it is considered that the amount of ROS in the liquid and the BA have a correlation from the formula (2). Thus, correlation coefficients R between the change of the a and the BA with changing the plasma gas temperature are shown by the following formula.

$$R(X_i, Y_j) = \frac{\sum (x_{i,T} - \bar{x}_i)(y_{j,T} - \bar{y}_j)}{\sqrt{\sum (x_{i,T} - \bar{x}_i)^2 \sum (y_{j,T} - \bar{y}_j)^2}} \quad (2)$$

Where $x_{i,T}$ is amount of introduced ROS i into solution by $T^\circ\text{C}$ plasma irradiation, $y_{j,T}$ is set of the BA on bacteria j at each plasma gas temperature, X_i and Y_j are set of $x_{i,T}$ and $y_{j,T}$, respectively, \bar{x}_i and \bar{y}_j are the mean values of the elements of set of X_i and Y_j respectively. The value of R approaches 1.00 if a positive correlation exists between the BA and the ROS quantity at the gas temperature of each plasma, and approaches 0 if irrelevant.

RESULTS AND DISCUSSION

Influence of plasma gas temperature on the bactericidal effect

Fig.2 shows the number of surviving bacteria irradiated with the temperature controlled plasma. In the irradiation of 10°C plasma on 200 μL suspension of *E. coli*, the initial number of which was 6.8×10^7 CFU/sample, the number of surviving *E. coli* decreased to 2.2×10^4 CFU/sample during 2 min. By increasing the plasma gas temperature above 50°C , the numbers of surviving *E. coli* in both cases decreased to less than the detection limit within 1/3 min. A similar trend with the bactericidal effect was confirmed for other general bacteria. For *B. cereus* with an initial bacterial number of 1.4×10^7 CFU/sample, the surviving number following plasma irradiation for 2 min decreased as the plasma gas temperature increased, and the surviving number when irradiated with 80°C plasma was 6.0×10^2 CFU/sample. In each graph, the time rags between the plasma irradiation and decreasing the number of the surviving bacteria were confirmed. The number of surviving *E. coli* maintained after the irradiation with 40°C plasma to the bacterial suspension containing L-histidine.

The BA values on *P. aeruginosa* were calculated at each gas temperature. The threshold value was 9.2×10^5 CFU/sample, since the initial number of *P. aeruginosa* was 9.2×10^7 CFU/sample. The upper dashed line in Fig.2 shows the threshold value, and the solid line connects the two points used to obtain the BA value. For example, the number of surviving *P. aeruginosa* irradiated 10 and 20°C plasma were 4.9×10^7 and 2.3×10^7 CFU/sample, respectively, the decreasing numbers were less than 1-order. Therefore, the BA values at these temperatures were 0. The number of surviving was less than the threshold value by 30°C plasma irradiation for 1 min, and was 6.7×10^4 CFU/sample for 2 min. Therefore, the BA value was $\log(2.9 \times 10^7 / 6.7 \times 10^4) / (2 - 2/3) = 2.0 \text{ min}^{-1}$. At 40°C , the number was less than the threshold value for 1/3 min, and was less than the detection limit for 1.5 min. Therefore, the BA value was $\log(9.2 \times 10^7 / 200) / (1.5 - 0) = 3.8 \text{ min}^{-1}$. At over 60°C , the number were less than the detection limit within 1/3 min, thus the BA value was $\log(9.2 \times$

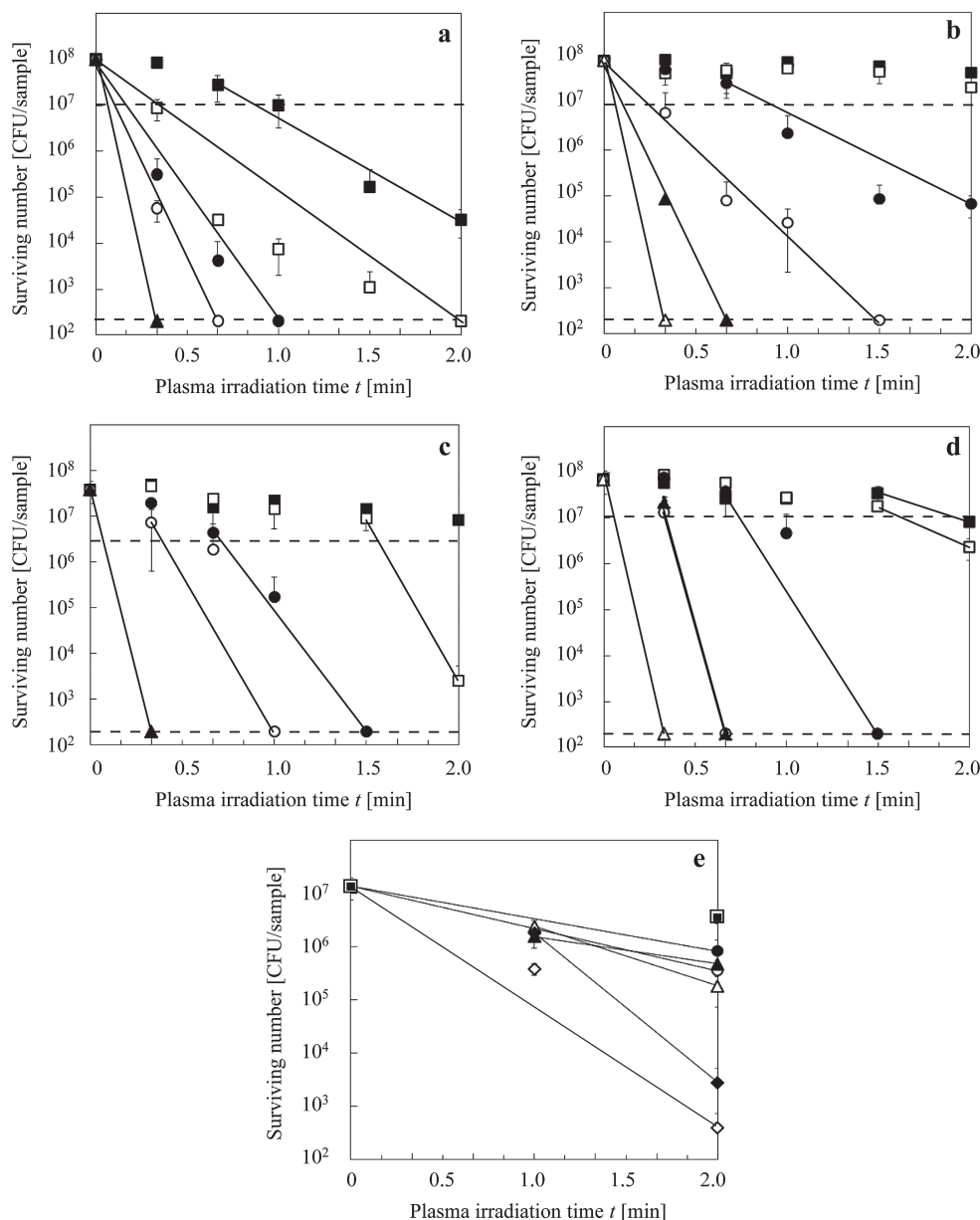


FIG. 2. Influence of plasma gas temperature on various kinds of bacteria. (a) *E. coli*. (b) *P. aeruginosa*. (c) *S. aureus*. (d) *E. faecalis*. (e) *B. cereus* (spore). 200 μ L of each bacterium suspension were irradiated with 3% oxygen mixed helium plasma controlled from 10 to 80°C. The surviving numbers of each bacterium were decreased with increasing the irradiation time and the plasma gas temperature. The lines were used for calculations of the *BA* values. The upper dotted line means the threshold value. The under dotted line means the detection limit at 200 CFU/sample. Symbols (plasma gas temperature): ■, 10°C; □, 20°C; ●, 30°C; ○, 40°C; ▲, 50°C; △, 60°C; ◆, 70°C; ◇, 80°C.

$$10^7/200) / (1/3) = 17 \text{ min}^{-1}.$$

Table 1 presents a summary of *BA* on each bacterium. The *BA* of 10-°C plasma irradiation to *E. coli*, *P. aeruginosa*, *S. aureus*, *E. faecalis*, and *B. cereus* were 2.0, 0, 1.3, 0 and 0 min^{-1} , respectively. By contrast, in the 80-°C plasma, the *BA* was >17, >17, >17, >16 and 3.0 min^{-1} , respectively. These results quantitatively show

that spore-forming bacteria have higher resistant to plasma than other bacteria and there is no significant difference between other bacteria. Additionally, these results quantitatively show that the *BA* on increases when the plasma gas temperature increases with respect to any type of bacteria.

To consider the contribution of heat on the bacteri-

TABLE 1. The calculated *BA* values of each bacterium at various gas temperatures. The *BA* values on each bacterium were calculated from the decreasing number of surviving bacteria and the time of the plasma irradiation. The values increased with increasing the plasma gas temperature.

Plasma gas temperature [°C]	<i>BA</i> [min ⁻¹]				
	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>E. faecalis</i>	<i>S. aureus</i>	<i>B. cereus</i>
10	2.6	0	1.3	0	0
20	3.3	0	1.8	7.1	0
30	5.5	2.0	6.4	5.2	0.61
40	8.3	3.8	14	6.8	0.80
50	>17	8.5	15	>16	0.52
60	>17	>17	>17	>16	1.1
70	>17	>17	>17	>16	2.8
80	>17	>17	>17	>16	3.0

cidal effect, each bacterial suspension should be treated with only heated gas at 80°C. We confirmed that no decrease in the number of surviving bacteria after only 80°C gas was blown on them for 2 min. In addition, the temperatures of DPBS treated with plasma of 10, 30, 60, and 80°C for 1 min were 6, 15, 20, and 23°C, respectively. The temperature became equilibrium in approximately 10 s after plasma irradiation. These results reveal that the direct contribution of the heat of plasma on the bactericidal effect was small. Therefore, we showed that the production of bactericidal factor increased with an increase in plasma gas temperature.

Influence of plasma gas temperature on ROS concentration

Fig.3 shows the concentration of HO·, ¹O₂, O₃, and H₂O₂. The concentrations were gradually increased according to the increase in plasma gas temperature. Through 10-°C plasma irradiation, the concentrations of HO·, O₃, and H₂O₂ were 34, 0.2, and 12 μM respectively. In addition, through 80-°C plasma irradiation, the concentrations were 72, 1.8, and 19 μM respectively. However, the concentration of O₃ at 70°C was lower than that at 80°C. The concentration of ¹O₂ rapidly increased with increasing plasma gas temperature: 18 μM for 10-°C plasma and 271 μM for 80-°C plasma.

By calculating *R* between *BA* of each gas temperature plasma and the amount of ROS introduced into the solution, the ROS involved in the bactericidal effect were investigated. The amount of ROS was calculated as the mole number introduced into the solution per minute. An example of the relationship between the *BA* values and amount of ROS was shown in Fig.4. The open squares are used as the points for calculating *R*, the closed squares are the excluded points. The points in the graph show the *BA* values on *P. aeruginosa* and the amount of introduced ¹O₂ at each plasma gas

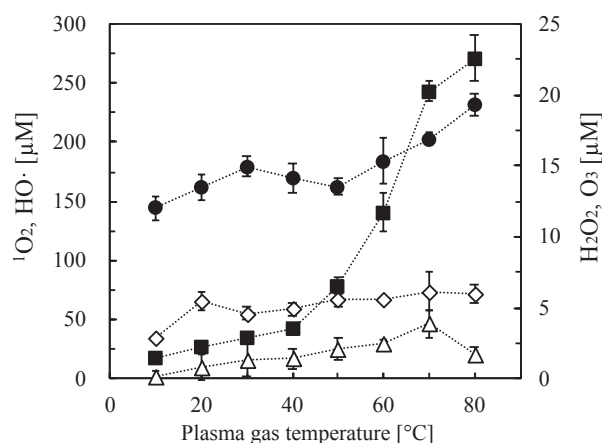


FIG. 3. Concentrations of ROS in a solution irradiated with plasmas of various gas temperature. 200 mM of 75 mM of DMPO and TPC were used as spin trapping reagent in ESR for the measurement HO· and ¹O₂, respectively. Indigo and XO were used in absorption spectrophotometry for the measurement of O₃ and H₂O₂, respectively. Solutions including reagent were irradiated with temperature controlled plasma for 30 s. The concentration of ¹O₂ and HO· were showed left vertical axis. The concentration of H₂O₂ and O₃ were showed right vertical axis. The concentrations of each ROS were changed with increasing the plasma gas temperature. Symbols: ■, ¹O₂; ●, HO·; ◇, H₂O₂; △, O₃.

temperature. In this case, the points between 20 and 60°C were used for calculating *R*. This *R* was 1.00 and very strong correlation was found between the *BA* on *P. aeruginosa* and the amount of introduced ¹O₂.

The values for *R* are listed in Table 2. As a result, very strong correlations were found (*R* = 0.80 to 1.00) between the change in the *BA* for each bacterium and the change in the amount of ¹O₂. Very strong correlations were then confirmed (*R* = 0.65 to 0.94) between the change in the *BA* for each bacterium and the change in

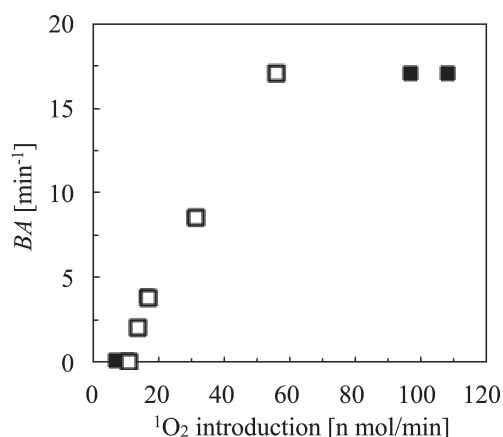


FIG. 4. Relationship between the amount of $^1\text{O}_2$ introduced into solution by plasma irradiation and BA value on *P.aeruginosa*. The amount of $^1\text{O}_2$ per minute was showed on the horizontal axis. The BA value on *P. aeruginosa* was showed on vertical axis. The correlation coefficient value R was 1.00. Symbols: \square , Used points for calculation of R between the amount of $^1\text{O}_2$ and BA value; \blacksquare , Excluded points.

TABLE 2. Determination coefficient (R) of the BA and ROS concentration accompanying change in plasma gas temperature. The R values between the BA values of each bacterium and concentration of $^1\text{O}_2$ were higher than which of other ROS.

Bacteria	R			
	$\text{HO}\cdot$	$^1\text{O}_2$	H_2O_2	O_3
<i>E. coli</i>	0.43	0.95	0.67	0.87
<i>P. aeruginosa</i>	0.58	1.00	0.53	0.94
<i>E. faecalis</i>	0.61	0.80	0.60	0.92
<i>S. aureus</i>	0.83	0.95	0.32	0.88
<i>B. cereus</i>	0.71	0.97	0.94	0.65

the amount of O_3 . A strong correlation was confirmed ($R = 0.43$ to 0.83) between the amount of $\text{HO}\cdot$ and the BA for each bacterium. A strong correlation also was confirmed ($R = 0.32$ to 0.94) between the amount of H_2O_2 and the BA for each bacterium. These results indicate that all ROS measured in this study were involved in bacterial inactivation. As a factor that the effective ROS varies depending on the bacterial species, there is a possibility that the differences in structure such as Gram negative and positive influence the bactericidal effect. In the ROS, the amount of $^1\text{O}_2$ and O_3 in 80°C plasma was higher than in 10°C plasma by 15 and 9.0 times, respectively. Thus, a high possibility exists that the $^1\text{O}_2$ and O_3 concentration generated by plasma irradiation contribute to the bactericidal effects on each bacterium.

Since the bactericidal effect was decreased in the

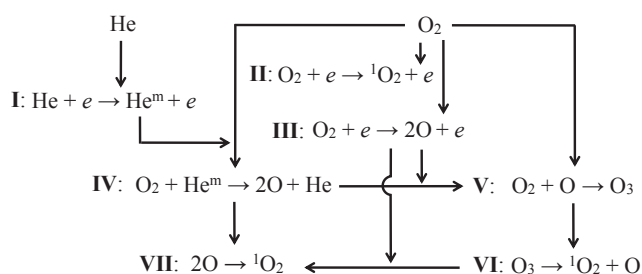


FIG. 5. Reaction route of $^1\text{O}_2$ productions. He and O_2 are excited by collision with electron (I), (II). O_2 is decomposed into atomic oxygens by collision with electron or excited He (III), (IV). O_3 is generated by recombination of O_2 and atomic oxygen (V). $^1\text{O}_2$ is generated by decomposing of O_3 (VI), and recombination of atomic oxygens (VII).

presence of L-histidine, the contribution of $^1\text{O}_2$ to the bactericidal effect was suggested. The lifetime of $^1\text{O}_2$ is very short on the order of microseconds in the liquid phase. A contradiction between the lifetime and the hypothesis of relational equation (1) was occurred. These findings suggest that a long-lifetime bactericidal factor is contributed to the bactericidal effect, and $^1\text{O}_2$ is a precursor of the bactericidal factor in the generation process.

$^1\text{O}_2$ is considered to be mainly generated by the reaction process shown in Fig.5. First, because of collision with electrons in the plasma, helium moves to a metastable state (He^m) (I) (Karakas et al., 2010), and oxygen molecules become $^1\text{O}_2$ (II) (Ionin et al., 2007) and are atomized (III) (Bian et al., 2013). In addition, the collision of helium in a metastable state with oxygen molecules causes atomization of oxygen molecules (IV) (Liu et al., 2010). The generated oxygen atoms combine with oxygen molecules, and O_3 is generated (V) (M. Laroussi and Leipold, 2004). The generated O_3 decomposes when encountering heat, and oxygen atoms and $^1\text{O}_2$ are produced (VI) (Ionin et al., 2007). However, oxygen atoms combine with each other to form $^1\text{O}_2$ (VII) (Ionin et al., 2007).

When the gas temperature of plasma is 50°C or higher, the following two factors are considered for the rapid increase in $^1\text{O}_2$ concentration. The first factor is an increase in gas flow velocity. As the gas temperature increases, the gas expands and the gas blowing speed increases. Therefore, the ROS generated in the reaction of IV to VI may reach the solution before inactivation. The second factor is the promotion of the decomposition rate of O_3 by heat. The liquid temperatures irradiated with the plasmas of 10 and 60°C for 60 s were 6 and 20°C , respectively. Therefore, the decomposition process of O_3 (V) was believed to have been promoted by the increase of the plasma gas and liquid temperatures. With the increasing amount of generated O_3 , the

reaction V was believed to have been enhanced by a rise in plasma gas temperature. However, the highest concentration of O₃ was measured in 70-°C plasma, whereas the concentration decreased in 80-°C plasma. This result indicates that the reaction VI was also enhanced by a rise in plasma gas temperature.

In this study, we revealed that the increased bactericidal effect was caused by generation of a greater number of high bactericidal ROS as the plasma gas temperature increased independently of the discharge power. A high efficiency bacterial inactivation can be realized by controlling the plasma gas temperature as high as possible. However, when a heat sensitive object is treated with plasma, the gas temperature should be controlled below the temperature which does not cause thermal damage to the object. These indicate that the gas temperature of the plasma should be controlled to an appropriate temperature. Therefore, we concluded that precisely controlling the gas temperature of the plasma is important for developing a new sterilization technique for plasmas.

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