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Bactericidal Action of Calcium Oxide Powder

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Using four antibiotics of which primary inhibitory actions were well understood, an investigation was made to determine the damage to *Escherichia coli* by the CaO powder slurry. The CaO treatment enhanced the sensitivities of *E. coli* to rifampicin and chloramphenicol. Although the CaO powder slurry is highly alkaline, alkaline treatment (NaOH solution with pH 11.0) killed the *E. coli* but not induced any changes in sensitivity to the antibiotics. It was suggested that the CaO powder obviously had the other antibacterial factors as well as the alkaline effect. The production of superoxide anion, which is one of active oxygen species, was observed from the CaO powder slurry previously. The tendency in the sensitivity changes in response to the CaO powder slurry nearly agreed with that of active oxygen treatment. These results suggest that the active oxygen produced from the CaO powder slurry is one of the primary factors in its antibacterial mechanism.

Key words: calcium oxide, antibacterial activity, antibiotics, active oxygen

1. INTRODUCTION

Recently, the utilization of inorganic materials having antimicrobial activity has attracted considerable attention as a novel methodology in biocontrol (1-3). Previously, the antibacterial activities of 26 metallic oxide powders were evaluated by monitoring a change in the conductance of the culture medium consequent on growth of bacteria (conductance method) (4). About ten powders were found to inhibit bacterial growth, and especially, magnesium oxide (MgO), calcium oxide (CaO) and zinc oxide (ZnO) powders exhibited strong bactericidal action. The CaO powder showed an efficacy against the spores of *Bacillus subtilis* as well, which have high resistivities to heat and various antibacterial agents (5). The generation of active oxygen species including superoxide (O_2^-) was observed from CaO powder slurry (6). However, there have been little understandings of its antibacterial mechanism.

In a previous work (7, 8), the injuries to bacteria caused by the antibacterial ceramics (MgO, CaO and ZnO) were assessed on the basis of the changes in sensitivities of the injured cells to four kinds of antibiotics. This method could be applied as a simple means inferring and classifying the influences and damage induced by physical and chemical stresses, because each stress induced different changes in sensitivity of bacterial cells to the antibiotics (9). In the case that the antibacterial metallic oxides are applied to prevent microbial pollution in food process, natural resources, such as heated shell powder or heated dolomite, will be desired as food additives. These can be orally taken to supply minerals and contains the CaO as the main component. Thus, in this work, the injuries to the bacteria caused by active oxygen were examined using the foregoing methodology, and an investigation was made to assess whether or not the active oxygen was related to the antibacterial activity of CaO powder.

2. MATERIALS & METHODS

2.1 Test organism

Escherichia coli 745, which is stored in the Tokyo Metropolitan Laboratory of Public Health,

Table 1. Experimental condition

$Ce(SO_4)_2$	1.0×10^{-5} M
DMSO	15%
pH	9.0 (phosphate buffer)
H_2O_2	310 μg/ml

was cultured in Brain Heart Infusion broth (Difco) at 37℃ for 24 h. The culture was suspended with saline to yield a final bacterial concentration of approximately 10^8 CFU/ml.

2.2 Treatment of active oxygen

Using cerium sulfate ($Ce(SO_4)_2$; Kishida Chemicals) as an oxidizing agent to make the system containing O_2^-, hydrogen peroxide (H_2O_2; Mitsubishi Gas Chemical) was decomposed to O_2^- as the following reaction (10).

$$H_2O_2 + Ce^{4+} \rightarrow O_2^- + 2H^+ + Ce^{3+} \quad (1)$$

$Ce(SO_4)_2$ had no effect on *E. coli* at the concentration lower than 1.0×10^{-5} M and was insoluble over that concentration (data not shown). The lifetime of O_2^- in aqueous solutions markedly depends on the pH (proton concentration). At neural to acid range, the generated O_2^- easily returns to H_2O_2 by the dismutation reaction (11).

On the other hand, O_2^- is chemically stable in non-protic solvents because the dismutation of O_2^- requires H^+. The *E. coli* used in this work can endure an alkaline treatment up to pH 10.5 over 60 min. Dimethyl sulfoxide (DMSO: Dojindo Laboratories) had no effect on the *E. coli* at the concentration not more than 15% (data not shown). Thus, using 0.05 M phosphate buffer (pH 9.0) and DMSO, the solution was prepared. The treatment condition is summarized in Table 1.

In the treatment, H_2O_2 was added to 0.05 M phosphate buffer saline (pH 9.0) including 15 % DMSO. The mixture solution of 10 ml was poured into a vial with an inner diameter of 32 mm, and $Ce(SO_4)_2$ was added to the mixture. After keeping the mixture in water bath for 1 h at 37℃, the bacterial suspension of 100 μl was pipetted into the vial, and the mixture was then shaken with 110 strokes/min at 37℃.

2.3 Treatment of calcium oxide powder slurry

CaO powder was purchased from Kishida Chemicals, and its mean particle size was 2.7 μm. The powder was heated at 180℃ for 20 min and suspended with physiological saline. The concentration of the CaO powder slurry was 2.5 mg/ml. The treatment of CaO powder slurry was carried out as described in the previous work (7).

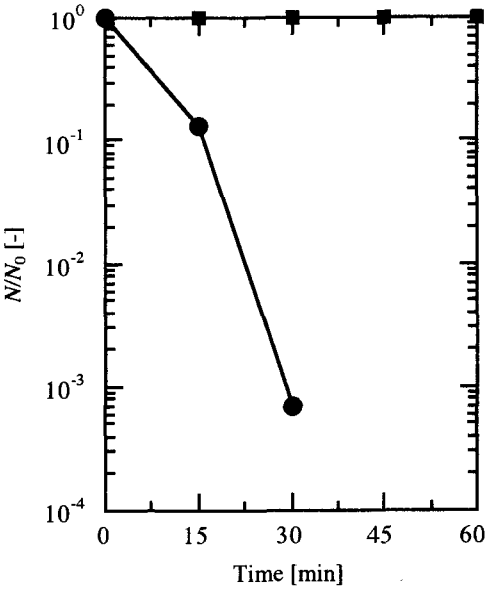

Fig. 1 Survival ratio of *E. coli* under the condition shown in Table 1 with (●) and without H_2O_2 (■).

Table 2. Antibiotics used here and their C_{MAX}.

Antibiotics	C_{MAX} [μg/ml]
Penicillin G (PCG)	14.8
Chloramphenicol (CP)	2.5
Nalidixic acid (NA)	1.0
Rifampicin (RFP)	2.2

2.3 Investigation of injuries of bacteria

The procedure is the same as described previously (7-9). After shaking, a 100 μl of sample was removed from the vial at a specified time and diluted with saline. Table 2 summarizes the antibiotics employed here as the selective reagents and their maximum concentration in an agar medium. The diluted sample was pour-plated with Sensitivity Disk Agar (Nissui Pharmaceutical). Duplicate plates were used for each dilution. The agar medium without antibiotics was used as the non-selective medium. Also, the agar media containing the antibiotics were used to detect injuries of the bacteria. After incubation at 37℃ for 48 h, the colonies were enumerated by using an automated colony-counter (Toyo CA-9).

3. RESULTS & DISCUSSION

Figure 1 shows the transient behavior of the

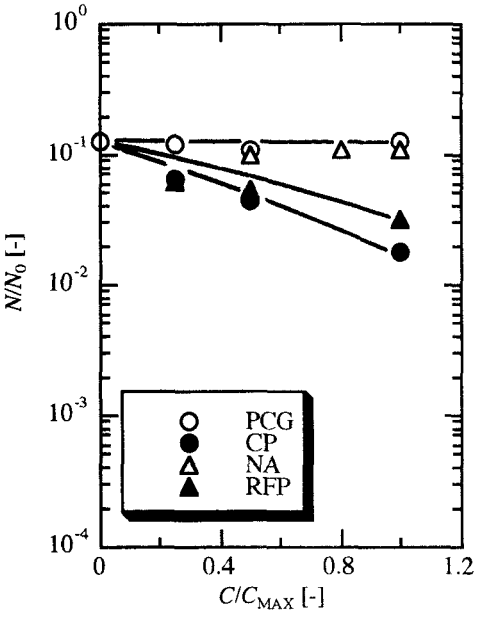

Fig. 2 Changes in sensitivity of *E. coli* to antibiotics by active oxygen treatment.

survival ratio (N/N_0) of *E. coli* in the system containing O_2^-. Without H_2O_2, the survival ratio of the *E. coli* did not decrease under the condition shown in Table 1.

Uninjured cells of bacteria can grow on both the selective medium and non-selective medium. However, some of the injured cells form colonies on the non-selective medium, but not on the medium containing the selective reagents, such as antibiotics. The difference in the colony counts with and without the antibiotic can be taken as a measure of the injury. When stress makes *E. coli* sensitive to an antibiotic, *E. coli* is thought to suffer some adverse influence corresponding to the sensitivity change.

Figure 2 shows the changes in the sensitivities of the *E. coli* to the antibiotics in the system containing O_2^-. The abscissa, C/C_{MAX}, represents the ratio of a specified concentration (C) to the maximum concentration (C_{MAX}) of the antibiotic in the agar medium. The C_{MAX} is the upper limit of the concentration at which the antibiotic has no effect on the colony formation of intact cells of *E. coli* and was determined previously (12). Incubation with the medium containing the antibiotic at the C_{MAX} will allow detection of a slight injury in the cells. As shown in Fig. 2, the N/N_0 on the media containing rifampicin (RFP) and chloramphenicol (CP) decreased with increasing C/C_{MAX}. This means that the treatments increased the sensitivities to RFP and CP. RFP acts on RNA polymerase and inhibits initiation of DNA-dependent RNA synthesis (13, 14). CP acts on ribosome (large subunit of 50 S) and

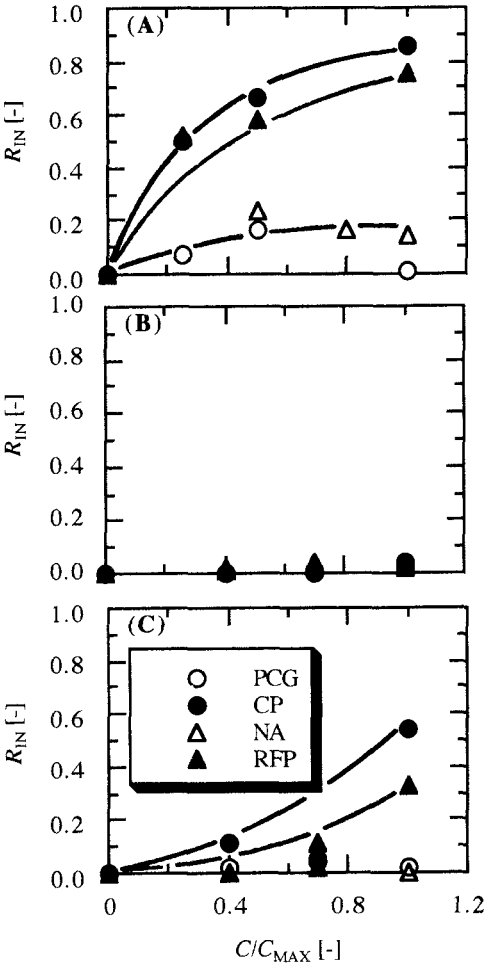

Fig. 3 Tendencies of sensitivity changes of *E.coli* induced by CaO powder slurry treatment at 2.5 mg/ml for 30 min (A), alkaline treatment (NaOH of pH 11.0) for 10 min (B), and active oxygen treatment under the condition for 15 min(C).

inhibits peptidyl transferase in protein synthesis (15, 16). These results suggest that the system containing O_2^- exerted some adverse influence on *E. coli* corresponding to the increase in the sensitivity to RFP and CP.

To discuss the antibacterial mechanism of CaO powder, the sensitivity change of *E. coli* treated by the system including O_2^- was compared to that by CaO powder or alkaline treatment (Fig. 3). R_{IN} is the ratio of the number of the injured cells to the number of the survivors and defined by Eq. (2).

$$R_{IN} = \frac{N(C/C_{MAX}=0) - N(C/C_{MAX})}{N(C/C_{MAX}=0)} = 1 - \frac{N(C/C_{MAX})}{N(C/C_{MAX}=0)} \quad (2)$$

The R_{IN} values for CaO and alkaline treatment were

calculated from the results obtained in the previous work (7) (Figs. 3(A) and (B)). The levels of N/N_0 on the non-selective medium for CaO and alkaline treatment were 3.7×10^{-2} and 1.3×10^{-1}, respectively. CaO powder slurry increased both the sensitivities to RFP and CP. The sensitivity change to the antibiotics by the system including O_2^- was similar tendency to that by CaO powder slurries, which generated O_2^- (Fig. 3(C)). Alkaline treatment did not increase any sensitivity to four kinds of the antibiotics (Fig.3(B)). When compared with alkaline treatment, CaO powder slurry has the antibacterial factor as well as the alkaline effect. Therefore, the active oxygen species will be one of the primary factors in antibacterial mechanism of the CaO powder slurry.

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