Effect of Diethylpyrocarbonate on *Lactobacillus casei* and Its Phage J1

Akira Murata, Ryohei Tanaka* and Toshinobu Kanegawa**

Laboratory of Applied Microbiology, Department of Agricultural Chemistry, Saga University, Saga 840, Japan

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Diethylpyrocarbonate (DEPC) exhibited a bacteriostatic effect at concentrations between 0.5 mM and 8 mM and a bactericidal effect at concentrations above 10 mM on *Lactobacillus casei* S-1. At the bacteriostatic concentrations the bacteria began to grow after the added DEPC had disappeared from the medium by self-decomposition, whereas at the bactericidal concentrations such growth was not observed even after long-term incubation.

DEPC inactivated free phage J1 within several min; 10, 20 and 30 mM of DEPC inactivated the phage by 90, 99.9 and 100%, respectively. The inactivation by DEPC was enhanced slightly by high temperature and markedly by low pH. Metal ions and chelating agents rendered negligible effects on the inactivation percentage.

DEPC did not cause strand scissions in the phage DNA, as exhibited by sucrose gradient centrifugation analysis. It changed the serum-blocking power of the phage, and this change was thought to be due to the structural alteration of phage tail-proteins.

The results are discussed in relation to the elimination of active phages from the dairy cultures.

In the dairy fermentations, chemical substances which are added into the lactic cultures are regulated to protect human being from noxious substances. To search for a harmless addition able to diminish active phages contained in the cultures, we have so far studied ascorbic acid,2,3) thiol compounds such as glutathione and cysteine,4–6) food preservatives officially permitted1,7–9) and β-propiolactone.10

Diethylpyrocarbonate (DEPC) is a substance which decomposes in aqueous solution into harmless components, carbon dioxide and ethyl alcohol.

\[ \text{C}_2\text{H}_5\text{O}-\text{CO} \xrightarrow{\text{H}_2\text{O}} 2\text{C}_2\text{H}_5\text{OH} + 2\text{CO}_2 \]

It has a strong bactericidal effect and has attracted the interest of the investigators who are engaging in the cold sterilization of beverages, especially soft drinks and wines.

Fedorcsák and Turtóczy11) and Kondorosi et al.11) have described that DEPC is a potential phage-inactivating agent. But, it appears that nobody has ever studied DEPC from the aspect of elimination of contaminated phages in the industrial fermentations, including the dairy fermentations.

In the present paper, as the first paper of a DEPC series, the effect of DEPC on *Lactobacillus casei* S-1 and its phage J1 are described. The effect of DEPC on the growth of phage J1 will be published elsewhere.13)

MATERIALS AND METHODS

*Bacterial and phage strains. *Lactobacillus casei* S-1, ATCC 27139, and its phage J1, ATCC 27139-B, were used throughout the experiments. A J1-phage-resistant strain derived from S-1 and a host-range mutant derived from J1 were also used in some cases.

*Chemicals.* Diethylpyrocarbonate (DEPC) with 100% purity was supplied by Dr. T. Matsuda of the Research Institute at the Ueno Fine Chemical Industries,
Ltd. Before each experiment DEPC was diluted in absolute ethyl alcohol to 100 times the desired concentration, and this 100 x DEPC solution was added to 9.9 ml of the reaction solution containing the bacteria or the phage at time zero. The concentrations of DEPC indicated in this paper are initial concentrations. Reaction solution contained 1% ethyl alcohol, but the effect of this concentration of ethyl alcohol on the bacteria and the phage was negligible. Other chemicals were from commercial sources.

**Medium and diluent.** MRT medium, pH 6.0,\(^{14}\) was used for growth of the bacteria and the phage. As a diluting fluid 0.01 m phosphate buffer, pH 7.0, containing 1 g NaCl, 0.25 g MgSO\(_4\)-7H\(_2\)O and 0.03 g gelatin per liter was used.

**Assay of bacteria and phage.** Viable cells were counted on agar plates.\(^{15}\) Plaque-formable phage was assayed by the double-layer method.\(^{14}\)

**Cultural condition.** Bacterial cultures were incubated without aeration at 37°C. The culture consisting of logarithmic phase cells was prepared by addition of an overnight-broth culture to 20 volumes of fresh broth and subsequent incubation for 3 ~ 4 hr.

**Turbidity measurement.** Turbidity of a bacterial culture in a Turbid-ell-cell, Fujimoto Rikaki, was measured at 620 nm in a Spectronic 20 spectrophotometer, Bausch and Lomb-Shimadzu.

**Chemical determinations.** Nucleic acids and proteins were fractionated as described by Schneider.\(^{16}\) Amounts of DNA, RNA and protein were determined by the diphenylamine method,\(^{17}\) the orcinol method,\(^{18}\) and the method of Lowry et al.,\(^{19}\) respectively.

**Serum-blocking power.** An antiphage serum was prepared by the method described previously.\(^{20}\) The experiment of serum-blocking power was mainly followed by the method of DeMars et al.\(^{21}\) The DEPC-treated phage was added to a diluted antiserum, K=0.02 min\(^{-1}\), and the mixture was incubated for 18 hr at 37°C. To the mixture was added a host-range mutant of phage J1 as a test phage. After 4 hr-incubation at 37°C survival of the test phage was assayed using a J1-phage-resistant strain as an indicator.

**Preparation of phage DNA.** An equal volume of buffer-saturated phenol was added to a purified phage suspension in 0.02 M Tris-HCl buffer, pH 7.3, and the mixture was shaken by hand for about 5 min. The aqueous phase containing DNA was separated by centrifugation and dialyzed against the same buffer. All operations were carried out in the cold.

**Sucrose density gradient centrifugation.** Linear 5 to 20% sucrose gradients, 4.6 ml, in 0.02 M Tris-HCl buffer containing 1 m NaCl (neutral, pH 7.3) or 0.2 M NaOH and 0.8 M NaCl (alkaline, pH 12.3) were prepared using a Mitumi density gradient former. A reaction mixture, 0.2 ml, was layered on the gradient, and the gradients were centrifuged in an RPS40A rotor of a Hitachi 55P-2 ultracentrifuge at 38,000 rev./min, 118,000 x g, for 240 min at 5°C. Three drops were collected for each fraction from the top of the centrifuge tube using a Mitumi SJ-1300UD gradient fractionator, to the each fraction was added 1 ml of water, and the absorbance at 260 nm was measured in a Hitachi 139 spectrophotometer equipped with a micro-cell (50 mm in length of optical path) attachment.

### RESULTS

**Effect of DEPC on growth of bacteria**

Various concentrations of DEPC were added to growing cultures of L. casei S-1, and the bacterial growth was followed by the measurement of turbidity and the colony-forming ability of cells.

**FIG. 1. Effect of DEPC on Growth Curve of Bacteria.**

Bacterial cells (2 x 10\(^8\)/ml) were incubated with DEPC at 37°C. Absorbance of the culture in a Turbid-ell-cell was measured in a Spectronic 20 spectrophotometer. Conc. of DEPC (mm): ---- 0; ◯, 0.5; ○, 1; ●, 2; △, 4; ▲, 6; □, 8; ■, 10 and 30.

**Figure 1** shows the effect of DEPC on the growth curve of the bacteria. At an initial concentration of 0.5 mm, DEPC rendered a
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small effect on the growth, but at higher concentrations up to 8 mM, DEPC produced a growth lag, and the lag period became longer with the increase in DEPC concentrations. After the lag, the turbidity increased at a reduced rate, but the ultimate turbidity was nearly equal to that of the DEPC-free control culture. Complete inhibition was achieved at 10 mM; the turbidity did not increase for a longer period up to 20 hr.

Table I shows the effect of DEPC on the viability (colony-forming ability) of bacterial cells. At 8 mM or below there was neither increase nor decrease in viable cell count during the lag period. The results showed that bacteriostatic action of DEPC in these concentrations. The viable cell count increased after the lag period. At 10 mM or higher there was decrease in viable cell count. The results showed the bactericidal action of DEPC in these concentrations. Once the viable cell count had reduced to zero, no increase in viable cell count occurred even after a longer period incubation up to 20 hr. This indicated that the killing by DEPC was complete.

The results coincide with the above observation that there is no increase in turbidity at 10 mM, but at 8 mM or below the turbidity increases after the lag.

In order to measure the time required for the complete decomposition of DEPC under the conditions of this experiment, DEPC was added to a fresh broth and the DEPC broth was preincubated before adding bacteria. Figure 2 shows the growth curves of bacteria added to the DEPC broth preincubated. The results indicated that DEPC decomposed completely in several min (cf. Fig. 4). The bacteria grew almost normally* when they were added after DEPC had completely decomposed.

Thus, the growth of bacteria after the lag in bacteriostatic concentrations (Fig. 1 and Table I) can be explained by the decomposition of DEPC into ineffective components. It should be noted, however, that the bacterial growth did not take place soon after the decomposition of DEPC, e.g., there was a lag of about 200 min at 8 mM.

**Effect of DEPC on macromolecular synthesis of bacterial cells**

Various concentrations of DEPC were added to growing cultures of L. casei S-1, and

![Diagram](image_url)

**Table I. Effect of DEPC on Colony-Forming Ability of Bacteria**

<table>
<thead>
<tr>
<th>Conc (mM)</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Incubation time (min)</td>
</tr>
<tr>
<td>0</td>
<td>0  60  120  180  240</td>
</tr>
<tr>
<td>1</td>
<td>100 130 190 290 430</td>
</tr>
<tr>
<td>2</td>
<td>100 110 140 220 320</td>
</tr>
<tr>
<td>3</td>
<td>100 100 130 190 260</td>
</tr>
<tr>
<td>4</td>
<td>100 100 120 160 230</td>
</tr>
<tr>
<td>6</td>
<td>100 100 100 110 150</td>
</tr>
<tr>
<td>8</td>
<td>100 100 100 100 115</td>
</tr>
<tr>
<td>10</td>
<td>100 90 65 25 10</td>
</tr>
<tr>
<td>20</td>
<td>100 0   --- --- 0</td>
</tr>
</tbody>
</table>

* A slight decrease in the growth rate of bacteria may be due to mainly the lowering of pH (cf. Fig. 6).
the net macromolecular synthesis of bacterial cells was followed. The results are shown in Table II. At bactericidal concentration, DEPC (10 mM) completely inhibited the synthesis of nucleic acids and protein for a long period. At bacteriostatic concentrations, DEPC completely inhibited the macromolecular synthesis during the lag period, but once the bacterial growth had started after the decomposition of DEPC the normal synthesis occurred. At lower concentrations of DEPC, the macromolecular synthesis continued without a lag but at a reduced rate, and the rate of DNA synthesis was more strongly reduced than those of RNA and protein syntheses.

**Effect of DEPC on free phage**

Figure 3 shows the effect of various concentrations of DEPC on free phage J1. At 3 mM DEPC had little effect on the phage. At higher concentrations DEPC inactivated the phage. The plaque-forming unit rapidly decreased for the first several min, and thereafter the decrease in the unit ceased. When concentrations of DEPC increased the degree of phage inactivation increased; 10, 15 and 20 mM of DEPC inactivated 90, 99 and 99.9%, respectively, of the phage. The phage at the order of $10^8$ PFU per ml was completely inactivated by 30 mM of DEPC in a few min. These data show that, among the substances known to have inactivating effect on phage J1, DEPC is the most powerful phage-inactivating agent.

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>Time incubated (min)</th>
<th>Relative amount (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>DNA 100, RNA 100, Protein 100</td>
</tr>
<tr>
<td>0</td>
<td>70</td>
<td>DNA 100, RNA 100, Protein 100</td>
</tr>
<tr>
<td>1</td>
<td>70</td>
<td>DNA 120, RNA 145, Protein 150</td>
</tr>
<tr>
<td>2</td>
<td>70</td>
<td>DNA 110, RNA 115, Protein 120</td>
</tr>
<tr>
<td>4</td>
<td>70</td>
<td>DNA 100, RNA 100, Protein 100</td>
</tr>
<tr>
<td>4</td>
<td>180</td>
<td>DNA 165, RNA 175, Protein 175</td>
</tr>
<tr>
<td>10</td>
<td>240</td>
<td>DNA 100, RNA 100, Protein 100</td>
</tr>
</tbody>
</table>

**Fig. 3.** Effect of DEPC on Free Phage. Phage ($2 \times 10^8$ PFU/ml) in MRT broth, pH 6.0, was incubated with DEPC at 37°C. Concentrations of DEPC (mM): ---, 0; •, 3; ●, 5; ○, 10; △, 15; ▲, 20; □, 30.

In order to confirm that the plateau after the initial decrease is due to the DEPC decomposition into ineffective components the following experiment was carried out. DEPC was added to a fresh broth, and the broth was preincubated before adding phage. As shown in Fig. 4, it was confirmed that DEPC decomposed completely within several min (cf. Fig. 2), and also that the plateau was explained by the DEPC decomposition.

**Fig. 4.** Effect of Preincubation of DEPC Solution on Inactivation of Phage. DEPC (10 mM) was added to MRT broth, and the broth was preincubated for the indicated times at 37°C. Then, phage ($2 \times 10^8$ PFU/ml) was added to the DEPC-broth, and this mixture was incubated further for 20 min at 37°C.

**Some factors affecting inactivation of phage by DEPC**

Some factors affecting the inactivation of
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phage J1 by DEPC were investigated.

Figure 5 shows the effect of temperature on the inactivation. The inactivation occurred even at 0°C. The rate of phage inactivation increased as the temperature was raised, but the degree of phage inactivation was nearly the same. The time necessary to reach the plateau was long at low temperatures.

Figure 6 shows the effect of pH on the inactivation of phage by DEPC. When pH was lowered the degree of phage inactivation increased; the degree was much greater at pH 4 than at pH 9. Thus, the inactivating effect of DEPC was strongly dependent on pH. It was noticeable that at alkaline pH there was a tendency to recover the plaque-forming unit during the prolonged incubation time. This indicates that the inactivation by DEPC may be reversible at alkaline pH.

Table III shows the effects of metal cations and chelating agents on the inactivation of phage by DEPC. Both monovalent and divalent cations tested rendered negligible effects on the inactivation percentage, and chelating agents also showed little effect.

**TABLE III. Effect of Metal Cations on Inactivation of Phage by DEPC**

<table>
<thead>
<tr>
<th>Cation</th>
<th>Conc. (mm)</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Na⁺</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>K⁺</td>
<td>100</td>
<td>9</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>0.1</td>
<td>11</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>0.1</td>
<td>11</td>
</tr>
<tr>
<td>Fe²⁺</td>
<td>0.1</td>
<td>11</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>0.1</td>
<td>9</td>
</tr>
<tr>
<td>Citrate</td>
<td>0.1</td>
<td>11</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.1</td>
<td>10</td>
</tr>
</tbody>
</table>

*As monovalent cation chloride was used, and sulfate was used as divalent cation.

**Effect of DEPC on DNA and protein of phage particle**

The double-stranded DNA isolated from phage J1 by the phenol method was treated with DEPC. The resulting DNA was analyzed by sucrose density gradient centrifugation in order to examine whether or not DEPC cause breaks in the DNA molecule. As shown in Fig. 7, the sedimentation pattern at alkaline pH showed that there were no materials sedimenting slower than the untreated control. This result indicates that DEPC cause neither
Alkaline Sucrose Gradient Centrifugation of DEPC-treated Phage DNA.

DNA (ca. 50 μg/ml) in 0.02 M phosphate buffer, pH 6.0, was incubated with 15 mM DEPC for 20 min at 37°C, under which condition ca. 99% of phage was inactivated. The resulting mixture was sedimented through sucrose gradient at alkaline pH (12.3). For other explanations, see MATERIALS AND METHODS.

--- DEPC-free control.

double-strand breaks nor single-strand breaks in the phage DNA.

Phage J1 was treated with DEPC, and the serum-blocking power (SBP) of the treated phage was measured in order to examine whether or not the DEPC treatment gave a damage to phage proteins, especially to the protein of adsorption organ. The results are shown in Table IV. The SBP values of treated phage varied from experiment to experiment. However, there was a tendency of decreasing the SBP value of DEPC-treated phage as the percentage of inactivation was increased. The decrease in SBP value was not so great as compared with the change in inactivation, e.g., a 99.9% -inactivated phage showed only a 10~30% decrease in SBP value. Thus, a part of the phage inactivated by DEPC can be explained by the damage of adsorption organ, resulting in failure to attach to cells.*

It is evident from the above results that the target attacked by DEPC in the phage particle is not DNA but proteins, though the greater part of the phage inactivation by DEPC remains so far unexplained.

**DISCUSSION**

A virulent and a temperate phage of *Bacillus subtilis* and phage T4 of *Escherichia coli* have been reported to be inactivated by DEPC. However, detailed study on the inactivation has not been published so far and the mechanism of phage inactivation by DEPC is unknown except that DEPC does not react with phage DNA. Kondorosi et al. have shown that the biological activity of transfectious DNA isolated from a *B. subtilis* phage is not altered after treatment with DEPC.

The present results have shown that phage J1 of *L. casei* is also inactivated by DEPC, and that DEPC does not cause strand breaks in the phage DNA, as exhibited by sucrose density gradient centrifugation analysis. These confirm the results of earlier workers.

The serum-blocking power experiments have indicated that DEPC reacts with the tail protein of phage J1, thus resulting in loss of adsorption capability. However, the damage of adsorption organ explains only a part of the phage inactivation by DEPC. The fact that SBP values of DEPC-treated phage vary from experiment to experiment would suggest that the damage of adsorption organ is a secondary reaction which occurs when the phage is already inactivated.

* DEPC does not give a damage to J1-phage receptors on bacterial cells, as will be described in a forthcoming paper.13)
DEPC is known to be a powerful protein-denaturing agent, resulting in the inactivation of enzymes. The reaction of DEPC with active hydrogenium of amino groups (carbethoxylation) in proteins may be responsible for the protein denaturation. Taking into consideration all of the present results and the known facts, it is clear that the structural alteration of phage proteins by carbethoxylation is responsible for the loss of infectivity.

The aim of the present study is to establish whether DEPC can be applicable for the control of phages in the dairy fermentations. DEPC did not inactivate phage at the concentrations which did not inhibit the growth of bacteria, and DEPC completely inhibited the growth of bacteria at the concentrations which inactivated over 90% of phage. Since DEPC exhibits no selective action in the phage J1-L. casei system, DEPC cannot be used in the conventional manner when fermenting culture is contaminated with phage. Advantageously, DEPC rapidly decomposed in broth medium, and bacteria grew almost normally when they were inoculated after DEPC had completely decomposed. DEPC can be therefore used for the inactivation of phage when culture medium (raw milk) is contaminated with phage.

Further discussion will be described in a separate publication in which the growth inhibition of phage J1 by DEPC will be reported.

Acknowledgement. We wish to thank Prof. M. Hongo, Kyushu University, and Prof. R. Saruno, this University, for their valuable discussions and encouragement. Thanks are also due to Dr. T. Matsuda, Ueno Fine Chemical Industries, Ltd, for his kind supply of diethylpyrocarbonate, Dr. T. Sakurai, Yakult Institute, for his kind supply of the phage and bacterial strains, and Miss K. Kitagawa for sucrose gradient centrifugation analyses. This work was supported in part by a research-aid-fund from the Kyushu Society for the Research of Microorganisms.

REFERENCES
3) A. Murata and K. Kitagawa, ibid., 37, 1145 (1973).