A Study on the Mechanism of DNA Excretion from *P. aeruginosa* KYU-1—Effect of Mitomycin C on Extracellular DNA Production—

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The mechanism of excretion of DNA was investigated using strain KYU-1. This strain produced a considerable amount of DNA extracellularly (7.8 mg/ml), and the amount of extracellular DNA per ml of the culture was scores of times more compared to that of intracellular DNA per ml of the culture. DNA synthesis was repressed by the addition of inhibitors (nalidixic acid, 5-fluoro uracil or mitomycin C), their inhibition rates were 35 to 54%. With the addition of 100 µg/ml of mitomycin C at the middle of the logarithmic growth phase, only 0.89 mg of extracellular DNA per ml of the culture was obtained, the inhibition rate was 95%. However, the amount of organic phosphate synthesized in the culture was almost the same no matter whether with or without an inhibitor.

Furthermore, the excretion of DNA from the cell surface of strain KYU-1 was observed with an electron microscope and the DNA molecules excreted were found to be double-stranded.

In a previous paper,1,2) the authors have reported that the strain KYU-1 grown on acetate as the sole carbon source produced a considerable amount of extracellular DNA. Moreover, DNA produced by this organism is active in transformation.3)

It has been known that various microorganisms excrete macromolecules such as protein and transforming DNA into the media, but less is known about the mechanism of DNA excretion. Extracellular DNA is found to originate from intracellular DNA and to be excreted under conditions in which lysis is not observed. However, the mechanism of the excretion of DNA by intact cells has not yet been understood. Therefore, a systematic study on the excretion of DNA by microbial cells as a whole may be valuable in revealing information on the excretion of extracellular macromolecules.

**MATERIALS AND METHODS**

**Microorganism.** The microorganism used in this study was *Pseudomonas aeruginosa* KYU-1,3) isolated from air as an acetate-utilizing organism in 1974, and preserved on nutrient agar slant in the author's laboratory.

**Culture conditions.** The medium for seed culture was composed of 1% CH₃COONa·3H₂O, 0.2% NH₄H₂PO₄, 0.2% (NH₄)₂HPO₄, 0.08% K₂HPO₄, 0.04% MgSO₄·7H₂O, 0.002% FeSO₄·7H₂O, 0.002% MnSO₄·4~6H₂O and 0.002% ZnSO₄·7H₂O, and was adjusted to pH 6.8. The seed culture (100 ml medium in a 500-ml flask) was sterilized at 115°C for 20 min, inoculated with the test organism and grown aerobically on a flask shaker (120 strokes/min; pitch 50 mm) at 30°C for 20 hr. The resulting seed culture (5 ml) was inoculated into 95 ml of the fermentation medium (3% CH₃COONa·3H₂O, 1% NH₄NO₃, 0.5% (NaPO₄)₆, 0.04% MgSO₄·7H₂O, 0.002% FeSO₄·7H₂O, 0.002% MnSO₄·4~6H₂O and 0.002% ZnSO₄·7H₂O; pH 6.8) in a 500-ml shaking flask and cultivated on a shaker at 30°C for 8 days.

**Analytical methods.** (1) Growth was determined by measuring the optical density (OD) at 660 nm of appropriately diluted culture broth and was converted to dry weight as described in the previous paper.1) (2) DNA samples of fibrous precipitate obtained by adding two volumes of 95% ethanol to the supernatant of culture broth were subjected to DNA assay by the method of Dische4) with calf thymus DNA as a standard. (3) RNA as a 75% ethanol-insoluble precipitate was determined by orcinol5) with yeast RNA as standard. (4) Intracellular nucleic acids were extracted and separated by the methods...
of Schmidt and Thannhauser⁶ and Schneider.⁷ (5) Acetic acid was estimated colorimetrically after steam distillation.⁸ (6) Organic phosphate was determined by the method of Allen.⁹ (7) For the assay of protein, the culture broth was centrifuged to remove the cells and an equal volume of 10% TCA was added to the clear supernatant. After allowing the product to stand for more than 30 min at room temperature, the precipitate was collected by centrifugation, dissolved with a small volume of 1 N NaOH and diluted to an appropriate volume with water. Protein was analyzed by the method of Lowry et al.¹⁰ with bovine serum albumin as a standard.

**Electron microscopy.** The cell preparation was mixed with an equal volume of 1 to 2% (w/v) potassium phosphotungstate (PTA) (pH 7.0), and placed on grids coated with collodion-carbon. Electron micrographs were taken by a JEM-7Y electron microscope (Japan Electron Optics Laboratory Co.).

**Nuclease digestion of DNA.** Digestion of DNA was carried out on suspensions of cells grown on acetate for 3 days at 30°C. The cells were washed twice with 1 mM Tris-HCl buffer (pH 7.9)–0.1 mM CaCl₂–0.25 mM sucrose, and resuspended in the same buffer at a cell concentration of 0.25 units at 660 nm. The kinetics of DNA digestion following the addition of nuclease was determined by measuring the amount of OD₂₆₀ absorbing material soluble in 1 M NaCl–1 M HClO₄. RNA contributed less than 5% of the total OD₂₆₀ as determined by treatment with pancreatic RNase. Nuclease reactions were terminated by the addition of EDTA to 5 mM.

**Chemicals.** Nalidixic acid, 5-fluoro uracil, mitomycin C and bacitracin were purchased from Boehringer Mannheim GmbH. Pancreatic DNase and pancreatic RNase were purchased from Boehringer Mannheim GmbH. Potassium phosphotungstate was purchased from Sigma Chemical Co. Other chemicals were of reagent grade.

**RESULTS**

**Time course of extracellular DNA production**

Figure 1 shows the time course of extracellular DNA production without pH-control of the culture. After a lag phase of 2 days, growth became visible and continued until the 4th day. When the acetate was largely consumed during the phase of growth and the pH of the culture rose to 9, DNA began to appear in the culture broth. The maximum yield was reached after 7 days and amounted to 7.8 mg per ml of the culture.

![Fig. 1. Time Courses of Extracellular DNA and RNA Production. The experiment was carried out under the conditions as described in MATERIALS AND METHODS. •, dry cell weight; ○, extracellular DNA; △, extracellular RNA; ■, pH; □, acetic acid.](image1)

During cultivation, as demonstrated in Fig. 1, the content of intra- and extracellular nucleic acids was estimated. As shown in Fig. 2, the amount of intracellular RNA was 0.37 mg per ml of the culture, while that of extracellular RNA was 0.3 mg per ml of the culture. It suggested that almost the whole intracellular RNA was accumulated in the broth by lysis. In the case of DNA, 0.1 mg of DNA per ml of the culture was present intracellularly, and the amount of extracellular DNA reached 7.8 mg.

![Fig. 2. Time Courses of Contents of Extra- and Intracellular Nucleic Acids in the Culture. The experiment was carried out under the same conditions as described in Fig. 1. •, intracellular DNA; ○, extracellular DNA; △, intracellular RNA; △, extracellular RNA.](image2)
The Mechanism of DNA Excretion from *P. aeruginosa* KYU-1

Table I. Effect of Various Inhibitors on Extracellular DNA Production

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>DNA (mg/ml)</th>
<th>Inhibition rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>4.57</td>
<td>0</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>3.21</td>
<td>35</td>
</tr>
<tr>
<td>5-Fluoro uracil</td>
<td>2.47</td>
<td>54</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>2.55</td>
<td>52</td>
</tr>
</tbody>
</table>

* Concentration added inhibitor was 10 μg per ml of culture broth.

Table II. Effect of Various Concentrations of Mitomycin C on Extracellular DNA Production

Cultivations were carried out for 6 days under the conditions as described in Materials and Methods, and then mitomycin C was added at the middle logarithmic phase.

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>DNA (mg/ml)</th>
<th>Inhibition rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.57</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>2.55</td>
<td>52</td>
</tr>
<tr>
<td>50</td>
<td>1.85</td>
<td>70</td>
</tr>
<tr>
<td>100</td>
<td>0.89</td>
<td>95</td>
</tr>
</tbody>
</table>

The amount of extracellular DNA was several scores compared with that of intracellular DNA.

Effect of inhibitors on extracellular DNA production

The effects of various inhibitors of 10 μg/ml on extracellular DNA production are shown in Table I. DNA synthesis was repressed by the addition of inhibitors and the inhibition rates were 35 to 54%.

The effects of various concentrations of mitomycin C and the time of its addition on extracellular DNA production were tested (Tables II and III). With the addition of 100 μg per ml of culture broth after 3 days, 0.98 mg of extracellular DNA per ml of the culture was obtained and its inhibition rate was 95%. The time for the addition of mitomycin C was important. If the inhibitor was added earlier, growth was more repressed and if the addition time was later, growth was less repressed. But in both cases, there were decreases in extracellular DNA production and its yields were decreased to 40 to 60% compared to that without inhibitor. The inhibition rates were 48.1 to 68.4% as shown in Table III. Furthermore, a 15% inhibition rate was observed even at the stationary phase. However, the amount of organic phosphate synthesized in the culture broth was almost the same whether it was with or without mitomycin C.

Effect of bacitracin on extracellular DNA production

Table IV shows the effects of bacitracin on extracellular DNA production.

Table III. Effect of Mitomycin C at Various Culture Periods on Extracellular DNA Production

The experiments were carried out for 6 days under the conditions as described in Materials and Methods.

<table>
<thead>
<tr>
<th>Culture period</th>
<th>DNA (mg/ml)</th>
<th>Inhibition rate (%)</th>
<th>Po&lt;sup&gt;+&lt;/sup&gt; (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>4.30</td>
<td>0</td>
<td>0.95</td>
</tr>
<tr>
<td>2 days</td>
<td>0.07</td>
<td>98.5</td>
<td>0.01</td>
</tr>
<tr>
<td>3 days</td>
<td>1.69</td>
<td>68.4</td>
<td>0.89</td>
</tr>
<tr>
<td>4 days</td>
<td>2.68</td>
<td>48.1</td>
<td>0.91</td>
</tr>
<tr>
<td>5 days</td>
<td>4.04</td>
<td>15.4</td>
<td>0.96</td>
</tr>
</tbody>
</table>

* Mitomycin C was added at a concentration of 50 μg per ml of culture broth.

b Organic phosphate.

Table IV. Effect of Bacitracin at Various Growth Phase on Extracellular DNA Production

Cultivations were carried out for 6 days under the conditions as described in Materials and Methods.

<table>
<thead>
<tr>
<th>Growth phase</th>
<th>Dried cell (mg/ml)</th>
<th>DNA (mg/ml)</th>
<th>Protein (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>3.40</td>
<td>3.33</td>
<td>1.59</td>
</tr>
<tr>
<td>Lag</td>
<td>2.36</td>
<td>2.58</td>
<td>2.28</td>
</tr>
<tr>
<td>Log</td>
<td>3.05</td>
<td>2.14</td>
<td>3.16</td>
</tr>
<tr>
<td>Stationary</td>
<td>3.40</td>
<td>2.21</td>
<td>3.18</td>
</tr>
</tbody>
</table>

* Bacitracin was added at a concentration of 50 μg per ml of culture broth.
extracellular DNA production. Apparently, bacitracin showed a marked stimulatory activity on the extracellular accumulation of protein. When bacitracin was added at the logarithmic phase of growth, the strain KYU-1 accumulated about 3 mg/ml of protein after 6 days cultivation, while the production of DNA was decreased to 60% by the addition of the drug.

**Excretion of DNA from the strain KYU-1**

The negatively stained electron micrograph is presented in Fig. 3. The excretion of DNA from the cell surface of the strain KYU-1 can be observed clearly in Fig. 3(a), while Fig. 3(b) shows the appearance of condensed DNA molecules. These phenomena strongly suggest that excreted DNA originates in the intracellular DNA molecules and might be in double-strand.

**Kinetics of nuclease digestion**

The results of the digestion of DNA excreted from the intact cells with pancreatic DNase show in Fig. 4. The cell suspension was susceptible to nuclease attack and a remarkable hyperchromic effect was observed during the nuclease action. However, no decrease at 660 nm was observed during incubation.

**DISCUSSION**

The occurrence of extracellular DNA in several bacterial cultures has been reported by Smithies and Bibbons and Catlin. The presence of transforming DNA in the cultures of bacteria such as *Neisseria*, *Pneumococcus* or *Bacillus* has been well documented. It is difficult, however, to exclude the possibility that these DNAs were released by the lysis of a small portion of the population. Therefore, the mechanism of transforming DNA excretion has been a subject of extensive studies because of the interest and importance of the problem, but its exact mechanism and role have remained unsolved.

The strain KYU-1 has the capability of producing a considerable amount of high polymerized DNA (MW 9.6 × 10^6 daltons) and DNA produced extracellularly is active in transformation. Dore et al. have reported that double strand DNA molecule was condensed at low
pH value (pH 1.9). In electron micrographs as shown in Fig. 3, the excretion of DNA synthesized near cell membrane outside the cell wall was observed, and condensed DNA molecules like a net were present. Furthermore, DNA produced by the strain KYU-1 extracellularly is capable of transformation, and the Tm value of extracellular DNA produced by the strain KYU-1 according to the method of Marmur et al. was 94.5°C. From these results, DNA molecules excreting outside cell wall are found to be double-stranded.

The inhibitors of DNA synthesis such as nalidixic acid, 5-fluoro uracil or mitomycin C were found to inhibit extracellular DNA production (Tables I, II and III). Nalidixic acid inhibits the DNA-polymerase reaction and thus inhibits replication, 5-fluoro uracil inhibits a certain step in the biosynthesis of deoxyribonucleoside triphosphates, and mitomycin C reacts with the DNA with formation of covalent bonds and then prevents the separation of the single DNA strands necessary for semi-conservative replication. This all shows that these inhibitors block the synthesis of DNA in cells.

There have been a number of studies on Bacitracin. Like other inhibitors of cell wall synthesis, bacitracin induces protoplast formation of bacterial cells and afterwards the cell lysis. Bacitracin caused a large increase in the production of exoprotein, but it did not cause any increase of the extracellular DNA production. Therefore, the mechanism of selective permeation might be present in the strain KYU-1 and then intracellular DNA can pass through cytoplasmic membranes without permanent damage to cells.

REFERENCES
4) Z. Dische, Mikrochemie, 8, 4 (1930).