MECHANISM OF CHLORAMPHENICOL RESISTANCE IN E. COLI

II. SENSITIVITY ALTERATION TO VARIOUS DRUGS OF CHLORAMPHENICOL RESISTANT E. COLI CONVERTED TO PROTOPLAST

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As was suggested in the first report of this series of work (Okamoto, 1959a), the CM* resistance of E. coli may depend, at least partly, on the permeability of the resistant cell. Our CM resistant strains of E. coli are also non-specifically resistant to penicillin (Pn), chlortetracycline (AM) and erythromycin (EM). If CM resistance originates from the non-specific impermeability of the resistant cells and, consequently, some alterations have occurred on the surface of such cells, influences on the resistance to CM and on those to three other drugs should be concomitant with each other. Attempts were made, therefore to test whether or not sensitivities will be altered more or less simultaneously, when the resistant cells convert to a form of the protoplast.**

Glycine treated protoplast was used in this report.

MATERIALS AND METHODS

Organism: Escherichia coli strain B054 and its chloramphenicol resistant strain CMR were used throughout this experiment. As was described in the previous report (Okamoto, 1959b), the resistant strain was obtained by the method of Szybalski (1952) and subcultured on agar slant containing 300 μg/cc of CM.

Chemicals: Chemicals used were the same as described in the first report (Okamoto, 1959a), except for glycine (Merck).

Medium: As a nutritional medium the following was used:

- Difco beef extract 0.3%
- Polypeptone (Daigo Eiyo Co.) 1.0%
- NaCl 0.5%
- It was adjusted to pH 7.0. In the case of agar plate, 1.2% of agar was added.

For the protoplast formation, the glycine medium was employed (Jeynes, 1957), where glycine in 3%, sucrose in 4% and MgSO₄ in 0.2% were added into the nutritional medium.

Protoplast formation: Protoplast of E. coli can be formed by a high concentration of penicillin. However, CMR did not form its protoplast even at a concentration of 100-200 u/cc of K-penicillin G. It may indicate that some differences exist between the cell wall of B054 wild type CMR.

* Abbreviations: chloramphenicol (CM), chlortetracycline (AM), erythromycin (EM), penicillin (Pn), chloramphenicol resistant strain (CMR), fifty per cent lethal dosis (LD₅₀), optical density (OD).

** As has been indicated by many authors (Brenner et al., 1958) E. coli does not convert to a real protoplast but to a "spheroplast". Since Jeynes (1957) no other work has shown that glycine treated cell is a "spheroplast", and therefore we call the "osmotically sensitive spherical form" used in this report "protoplast".

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and that of CMR strain. Therefore, either CMR or BO54 wild strain was treated with glycine medium. In a preincubation BO54 and CMR were rocked for 7 hours in a revers-T type of tube (1.8 cm of diameter) at 37°C for the optical density (OD) to reach about 0.5 at 650 nm. One cc aliquot of this bacterial suspension was added to 9 cc of glycine medium which was incubated for additional 4 hours in the same tube. After this glycine treatment, the cell suspension was divided into two, one was diluted with a 4% solution of sucrose and the other with distilled water. The sample diluted with 4% sucrose solution was plated on normal agar containing sucrose in 4%, and the other diluted with water was plated on normal agar without sucrose. The number of colonies developed on both sorts of agar was enumerated after the incubation for 24–48 hours. (Glycine protoplasts were found to be most stable in 4% of sucrose contrary to the previous works (Lederberg, 1956) where 20% of sucrose was used.) The number of protoplasts was calculated by deducting the number of colonies appearing in the agar plate without sucrose from that in the plate with sucrose. As shown in Table 1, the total number of colonies in the sucrose plate including intact as well as reversed cells reached 10% of the number of cells found before the glycine treatment. Thus, the drug sensitivity of protoplast was expressed by the reversibility from protoplast to rod in the presence of drugs at a concentration to be tested. The fifty percent lethal dose could be calculated by the percentage of the reversibility taking the protoplast without drug as 100%. The rod cells which did not form protoplasts after the glycine treatment showed the same sensitivity to the drugs as did the intact cells.

RESULTS

The Formation of Protoplasts by Glycine Treatment

As shown in Table 1, the cell number of both BO54 and CMR was about 10^8 per cc at the beginning of the glycine treatment. The number of protoplasts, calculated as described under Materials and Methods, is shown in the column (3) in Table 1. The morphology of the protoplasts was observed immediately after the glycine treatment ended by making a hanging drop sample (Fig. 1).

<table>
<thead>
<tr>
<th>Name of strain</th>
<th>No. of experiment</th>
<th>Inoculation size</th>
<th>No. of colonies appearing on agar plate with 4% sucrose (1)</th>
<th>No. of colonies appearing on agar plate without sucrose (2)</th>
<th>No. of protoplasts (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>1</td>
<td>2.1×10^8</td>
<td>3.2×10^7</td>
<td>1.6×10^7</td>
<td>1.6×10^7</td>
</tr>
<tr>
<td>BO54</td>
<td>2</td>
<td>—</td>
<td>1.1×10^7</td>
<td>5.7×10^4</td>
<td>5.3×10^4</td>
</tr>
<tr>
<td>E. coli</td>
<td>3</td>
<td>1.0×10^8</td>
<td>7.3×10^6</td>
<td>2.2×10^4</td>
<td>5.1×10^6</td>
</tr>
<tr>
<td>BO54-CMR</td>
<td>4</td>
<td>—</td>
<td>5.6×10^4</td>
<td>1.9×10^4</td>
<td>3.7×10^4</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>—</td>
<td>1.2×10^4</td>
<td>4.3×10^3</td>
<td>7.7×10^3</td>
</tr>
</tbody>
</table>

The cells were treated with glycine medium to form a protoplast suspension, including intact cells. This mixed suspension was plated on agar with or without sucrose. The number of protoplasts was calculated by the following equation:

No. of protoplasts (3) = No. in column (1) − No. in column (2)

Details are given in the text.

The Resistance of Protoplasts to CM

The reversibility of protoplasts with or without CM was compared with each other, taking the reversal in the absence of CM as 100%. One of the typical results is shown in Fig. 2. As a control the resistance to CM of BO54 wild strain, either in the-
Fig. 1. Glycine treated protoplasts of *E. coli* strain B054 and CMR. Stained with acetocarmine.

\[ \text{a: B054, b: CMR.} \]

Form of intact cells or of protoplasts, is also shown. LD\(_{50}\) of each case was as follows:

- **B054 wild type, intact cells**: 2.4 \(\mu g/cc\)
- **B054 wild type, protoplasts**: 1.8
- **B054 CMR, intact cells**: 1650.0
- **B054 CMR, protoplasts**: 450.0

It will suggest that B054 CMR in the form of protoplasts can be more sensitive to CM than the intact cells.

As is evident in Fig. 2, whereas the intact cells of CMR were resistant to 300 \(\mu g/cc\) the protoplasts showed 60% survival at the same concentration of CM. This means that 60% of the protoplast population was resistant to 300 \(\mu g/cc\) of CM. Therefore, the cells reversed from the protoplasts in this population should be resistant to CM.

Fig. 2. The alteration of sensitivity to CM depending on the cell conditions for *E. coli* wild and CMR strains.

The survival percentage was expressed taking the number of colonies in the absence of CM as 100%. The number of colonies reversed from the protoplast was calculated by the equation given in Table 1. Details are given in the text.
even after another trial of glycine treatment. Ten colonies randomly selected from this population were treated individually by glycine and tested likewise for the resistance to CM by the reversibility from the protoplast to the rod. The resistance expressed by the reversibility percentage showed a fluctuation between 87% to 110% in the presence of 300 μg/cc of CM. The arithmetic mean of this value was 93%.

**The Sensitivity to Other Drugs**

As reported in the previous paper (Okamoto, 1959a), the B054 CMR strain showed a cross resistance to Pn, EM and AM. Attempts were made to test the reversibility of the protoplast in the presence of these drugs. The same technique was applied to these drugs for the estimation of LD₅₀. Table 2 shows the results. A remarkable difference of LD₅₀ between the intact cells and protoplasts was observed only in the cases of EM and CM.

Table 2. LD₅₀ of various drugs for intact cells and protoplasts of *E. coli* wild and CMR strains

<table>
<thead>
<tr>
<th>Organism</th>
<th>Drugs</th>
<th>CM μg/cc</th>
<th>EM μg/cc</th>
<th>AM μg/cc</th>
<th>Pn μg/cc</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>Intact cell</td>
<td>2.4</td>
<td>42</td>
<td>3.4</td>
<td>14</td>
</tr>
<tr>
<td>B054 wild</td>
<td>Protoplast</td>
<td>1.8</td>
<td>18</td>
<td>1.8</td>
<td>3</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Intact cell</td>
<td>1650</td>
<td>2900</td>
<td>27</td>
<td>75</td>
</tr>
<tr>
<td>B054 CMR</td>
<td>Protoplast</td>
<td>450</td>
<td>160</td>
<td>15</td>
<td>48</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The sensitivity to CM of the CMR protoplast was examined in the present experiments by testing the reversibility of the protoplast to the rod cell. If the sensitivity remained constant before and after the protoplast formation, one can not tell whether the drug resistance can be attributed to the inability of the drug to reach the sensitive site of the cell or to some intrinsic resistant mechanisms involved in the protoplast. In the present experiments the protoplast showed in most cases a lower resistance to the drugs, indicating that the drugs could, at least penetrate the protoplast membrane of some cells and that they could, in turn, interfere with the metabolism of the protoplast.

If the cell wall of the CMR was impermeable to the drug, it is to be anticipated that the protoplast of the cells becomes less resistant than the intact cells to a given concentration of the drug. The results actually demonstrated that the protoplast of *E. coli* B054 was more sensitive to CM than the intact cells. Recently, Ramsey (1958) showed that the induced nitrate reductase was formed in CMR of *Staphylococcus* even in the stage of "disrupted cells". Since "disrupted cell" maintains some problems to be solved (Gale et al., 1958), his work is not discussed in the present paper. As shown in Fig. 1, 100% population of intact cells (CMR) were resistant to 300 μg/cc of CM while with protoplasts 60% of their population was resistant to the same concentration of CM (on the other hand, the difference in a similar calculation between the intact cells of wild *E. coli* and its protoplasts was negligible. (Fig. 2 and Table 2,
column 2). These resistant colonies derived from protoplasts were arbitrarily selected and examined individually for another trial of glycine treatment to test its reversal to rods in the presence of the drugs. Ten individual colonies showed 93% (mean value of 10 trials) population of the resistance. This indicates that two populations existed among the protoplasts, one was sensitive to CM occupying 40% and the other still resistant to 300 μg/cc of CM occupying 60%. That is to say, CM resistance of E. coli BO54 evidently depended partly on the cell wall permeability. However, the same fact will tell that this 60% of the protoplasts still maintained some intrinsic mechanisms of resistance apart from the cell wall permeability.

One additional point should be noticed in this connection. E. coli can form the protoplast in the presence of a high concentration of Pn. CMR of E. coli BO54 did not develop the protoplast even in the presence of Pn ranging from 100 to 2000 u/cc. Taking the mechanism of Pn action (Lederberg, 1957, Park and Strominger, 1957) into consideration, the above fact can be interpreted to suggest that there existed some differences in the nature of the cell wall between the wild and CMR strains.

Table 2 summarizes the LD₅₀ values of various drugs to the intact cell and the protoplast. The difference between intact cells and protoplasts with the wild strains was not so marked except in the case of Pn, whereas the differences with CMR strain was remarkable in the cases of CM and of EM. That is to say, the resistances to CM and to EM can be explained partly by the permeability of cell wall. But these varieties of the differences will show that the resistance is still due to some specific mechanism acting within the protoplast including the protoplast membrane. Thus, simultaneous alteration of the drug sensitivities was not observed in the present experiments. In short, the present report has provided an evidence that the CM resistance of E. coli BO54 derived partly from the CM impermeability of its cell wall.

**SUMMARY**

In an attempt to elucidate the cell wall permeability of chloramphenicol resistant E. coli BO54, the protoplast was developed by glycine treatment and tested for its reversal to the rod cell in the presence of chloramphenicol. Some alterations of sensitivities in the protoplast were observed, suggesting that the resistance partly depended on the cell wall permeability and that a considerable resistance still remained within the protoplast. Penicillin, chlortetracycline and erythromycin, to which the strains were cross-resistant, were also tested for the inhibition of the reversal from the protoplast to the rod cell.

**REFERENCES**


