CHARACTERIZATION OF SPONTANEOUS FOSFOMYCIN (PHOSPHONOMYCIN)-RESISTANT CELLS OF *ESCHERICHIA COLI* B *IN VITRO*

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(Received for publication June 2, 1975)

The characteristics of spontaneous fosfomycin-resistant cells isolated *in vitro* were investigated. Distribution of resistance level to the drug in *Escherichia coli* B was thought to be broad and, for this reason, resistance to the drug seemed to develope easily *in vitro*.

In the process of isolating the resistant cells, two groups of cells, differing in colony size, were distinguished. Smaller colony-forming cells appeared more frequently than Larger colony-forming ones. Many of the former seemed to be slow growers and decreased simultaneously in utilization of several carbohydrates. One of the Smaller colony-forming isolates was distinctly different from *glp* T^- or *uhp*$^-$.

Fosfomycin, produced by several *Streptomyces* species, is an acidic, water-soluble and cell wall-active antibiotic with a broad antibacterial spectrum.[1] It was reported by KAHAN *et al.* that the site of action of the drug was the initial step of cell wall biosynthesis and the target enzyme was phosphoenolpyruvate: UDP-GlcNAc-3-*O*-enolpyruvyl transferase in bacterial cytoplasm.[2] In this respect, penetration of fosfomycin into the cytoplasm is essential for the drug to exhibit its bactericidal activity, in contrast to β-lactam antibiotics which interfere with the last step of cell wall biosynthesis outside cytoplasmic membrane.

KAHAN *et al.* have established that entry of fosfomycin into the cytoplasm is mediated by L-α-glycerophosphate or hexose phosphate transport system.[2]

It was anticipated that the mutants defective in those transport systems would be selected as the fosfomycin-resistant cells *in vitro*. In fact, mutants defective in utilization of L-α-glycerophosphate were isolated as the fosfomycin-resistant strains,[2] and those defective in L-α-glycerophosphate transport system (designated, *glp* T^-) were resistant to fosfomycin.[2] Further, mutants defective in hexose phosphate transport system (designated, *uhp*$^-$) were isolated as the drug-resistant strains.[3]

It was reported that fosfomycin was effective in protecting mice against a variety of infections,[4] while resistance to the drug developed rapidly *in vitro* due to spontaneous mutation.[5] The characteristics of the spontaneous fosfomycin-resistant cells isolated *in vitro* have been investigated, as the first step to elucidate relationship of their development *in vitro* to *in vivo* activity of the drug, in our laboratory.

In this paper, the isolation process and the *in vitro* characteristics of fosfomycin-resistant strains, FR 90 and FR 95, are presented.

Materials and Methods

1. Bacteria. *Escherichia coli* B (*E. coli* B, IAM 1268) was used.

2. Culture media. Nutrient Broth (NB, Difco Lab., Detroit, U.S.A.) and Nutrient Agar (NA, Difco Lab.) were used. Media to examine carbohydrate utilization were EMB and MacConkey agar media (lactose replaced with one of various carbohydrates), and M_9 medium[6] (glucose replaced with one of various carbohydrates), as synthetic medium. Synthetic agar medium contained agar (Oxoid Ionagar No. 2, Oxoid Ltd. London, England) at 1 %.

3. Growth of cells. Bacteria maintained on an NA agar slant were inoculated into NB and incubated with shaking at 37°C. The overnight culture (0.1 ml) was inoculated into NB (10 ml) in an L-shaped test tube (a modified Monod test tube, 40 ml), unless otherwise noted, and it was incubated. Growth was monitored by reading the absorbancy at 550 nm (A_{550}) using the spectrophotometer (Bausch & Lomb Spectronic 20, Shimazu Seisakusho, Co. Ltd., Kyoto, Japan). It was shown that A_{550}'s were correlated linearly with cell densities up to 0.3 in A_{550}.

4. Distribution of fosfomycin-resistance level. An aliquot (0.5 ml) of the overnight culture was inoculated into NB (10 ml) containing disodium fosfomycin (FOM) at 10 μg/ml. Another aliquot was spread on NA plates containing fosfomycin at various concentrations after appropriate dilutions with NB. The inoculated liquid culture was incubated and an aliquot of the stationary phase culture was spread on plates by the above-mentioned procedure. The colonies on the plates incubated for 2~4 days were counted.

5. Isolation of fosfomycin-resistant cells. The culture inoculated with overnight culture into NB (10 ml) containing FOM at 10 μg/ml was incubated until stationary phase. The cells grown were subsequently transferred to fresh NB containing the drug at 50 μg/ml and then 100 μg/ml. Then the four sequential transfers to NB containing the drug at 100 μg/ml were done. The final stationary phase culture was diluted with NB and spread on NA plates containing FOM at 100 μg/ml. After incubation of the plates for 2~4 days, fosfomycin-resistant cells were isolated and master plates were prepared.

6. Examination of carbohydrate utilization. Colonies on the master plates were replicated on indicator plates which contained indicators and were supplemented with either L-arabinose, glucose, glycerol or disodium glucose-6-phosphate (G-6-P) at 1 %, and on the synthetic agar medium containing disodium D,L-α-glycerophosphate (α-GP) at 0.4 %. After incubation of these plates, utilization of the carbohydrates by the isolates was determined by observation of the replicated clones for 3 days. Each carbohydrate utilization of FR 90 and FR 95 was determined by observation of the growth for 10 hours on M_9 medium supplemented with one of the following carbohydrates at 0.4 %; L-arabinose, galactose, glucose, glycerol, α-GP, G-6-P, lactose, or mannitol.

7. Reagents. Disodium fosfomycin (FOM) ((−)-*cis*-1,2-epoxypropyl-phosphonate, 75 % (w/w) as a free acid) was a gift of Merck Sharp and Dohme Res. Lab., Rahway, N. J., U.S.A. G-6-P was purchased from Boeringer Mannheim GmbH, Denmark, α-GP from Nakarai Kagaku Co. Ltd., Kyoto, Japan, and sodium 3′,5′-cyclic AMP (c-AMP) from Sigma Chemical Co., St. Louis, U.S.A.

Results and Discussion

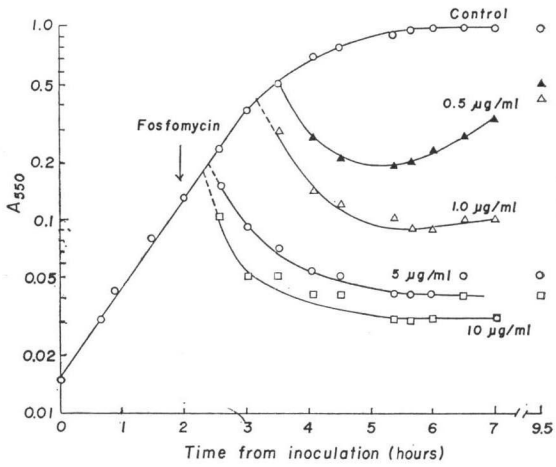
1. Development of Fosfomycin-resistant Cells

Fig. 1 shows the representative growth inhibition pattern of *E. coli* B by fosfomycin. When fosfomycin was added to the culture at the logarithmic growth phase, the decrease in A_{550} owing to cell lysis was observed. Exposure of the cells to as little as 0.5 μg/ml of FOM resulted in decrease in A_{550}, however, the absorbancy began to increase again after several hours. The later increase in A_{550} was common in each concentration of FOM shown in the figure; heavy growth occurred at 24 hours in the culture with the drug at 10 μg/ml. The cells from each recovered culture were no longer lysed by the addition of the drug at the concentration which had been added to the corresponding culture. These results indicate that fosfomycin-resistant cells grew in the cultures because of the addition of the drug.

The distribution of fosfomycin-resistant cells was examined. The resistant cells were isolated at a frequency of 10^{-1} in the selection on the NA containing the drug at 10 μg/ml and the highly resistant ones were obtained at a frequency of $10^{-7}\sim10^{-6}$ by the selection with FOM at 50 μg/ml. When the cells were grown in NB containing the drug at 10 μg/ml, the more highly resistant cells were present in the culture at a higher frequency. For example, those grown on NA containing the drug at 50, 100, or 250 μg/ml were present at the frequency of $10^{-4}\sim10^{-3}$, $10^{-5}\sim10^{-4}$ or $10^{-7}\sim10^{-6}$, respectively. From these results, it is suggested that the cell distribution with fosfomycin-resistance level is originally broad and, for this reason, the highly resistant cells are enriched easily by cultivation in the presence of the drug.

Fig. 1. Effect of fosfomycin on the growth of *E. coli* B.

Overnight culture was inoculated into fresh NB and incubated with shaking at 37°C. At mid logarithmic growth phase (shown by arrow), various concentrations of disodium fosfomycin (FOM) were added to the cultures.



2. Isolation Process of the Resistant Cells

In the process of isolation of the resistant cells, two groups, distinguishable groups in colony size, were found on the isolation plates. One group of smaller colonies (Smaller colony) and the other group (Larger colony) exhibiting colony size indistinguishable from the parental strain (sensitive strain) developed. The former usually appeared preferentially at the early transfers in the NB containing FOM at 100 μg/ml. For example, the ratio of the former to the latter was about 10 at the 2nd transfer, and the ratio became almost unity after several transfers.

The resistant isolates were examined for utilization of carbohydrates described in Materials and Methods by the replica method. Many of Smaller colony-forming isolates fell into two classes; those which seemed to be decreased simultaneously in utilization of several carbohydrates including α-GP but not G-6-P, and those which seemed to be decreased in use of α-GP only. Many of the Larger colony-forming ones seemed to be decreased in utilization of α-GP only. Some of fosfomycin-resistant isolates were also decreased in use of G-6-P and some others failed to grow on MacConkey, or MacConkey and EMB agar media. In one case, about 50 % (43 isolates) of Smaller colony-forming isolates (79) were decreased simultaneously in utilization of several carbohydrates including α-GP, about 40 % (33) of them were decreased in that of α-GP only, and the remainders (3) were not examined because they failed to grow on the indicators. While, about 90 % (37) of Larger colony-forming isolates (40) were not decreased in the utilization except α-GP and the remainders were decreased simultaneously in use of several carbohydrates including α-GP. In *E. coli* K-12 Hfr (Broda 1) *met*$^-$ (ATCC 23743), about 80 % (31) of Smaller colony-forming isolates (39) were decreased simultaneously in utilization of several carbohydrates including α-GP, and the remainders were decreased in use of α-GP only. In the

Fig. 2. Sensitization of fosfomycin-resistant strains to fosfomycin by G-6-P.

An overnight culture was inoculated at 2% into NB containing 50 μg/ml disodium fosfomycin, 100 μg/ml of the drug, 200 μg/ml of the drug, 50 μg/ml disodium G-6-P or 50 μg/ml of the drug+50 μg/ml disodium G-6-P.

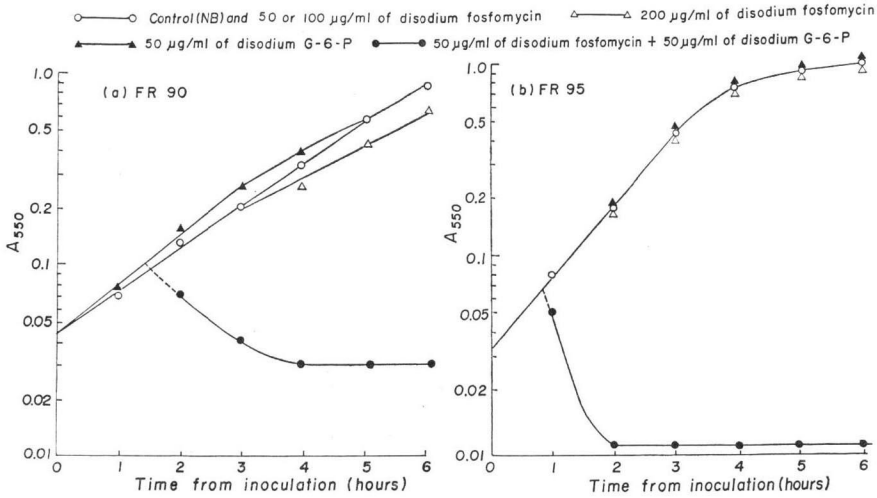
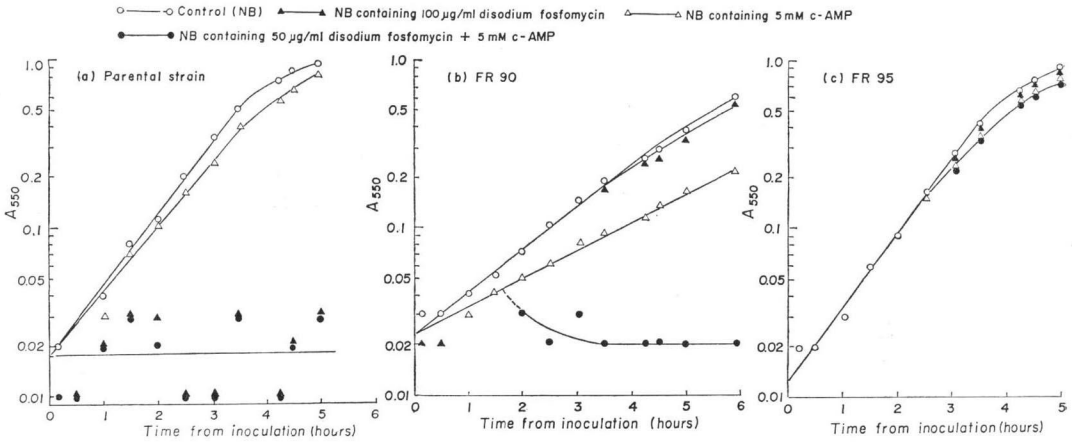


Fig. 3. Effect of c-AMP on the resistant strains.

An overnight culture was inoculated into NB containing 100 μg/ml disodium fosfomycin, 5 mM c-AMP or 50 μg/ml of the drug+5 mM c-AMP.



Larger colony-forming ones, 100% (39) were decreased in use of α-GP only.

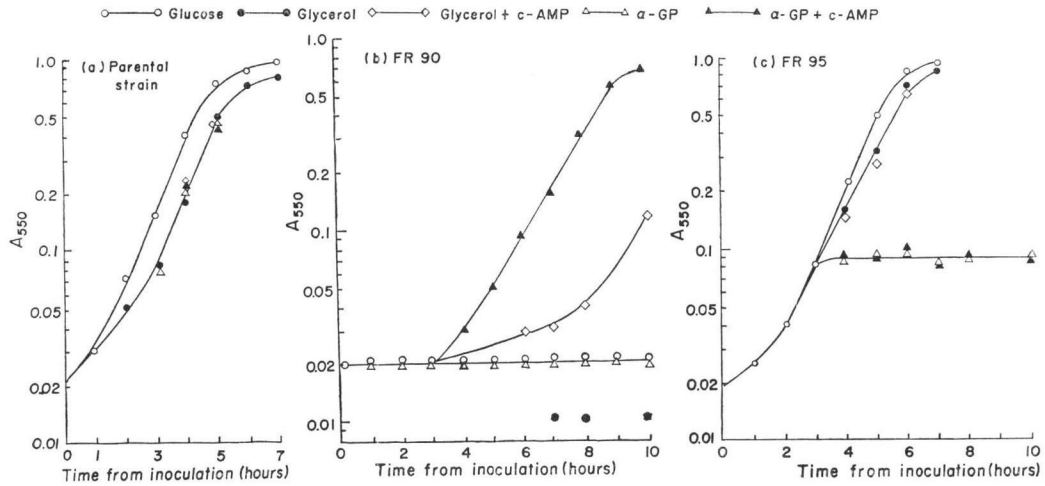
3. Characteristics of the Resistant Strains, FR 90 and FR 95

On the basis of the above results, FR 90 which formed Smaller colony and was decreased simultaneously in utilization of several carbohydrates, and FR 95 which formed Larger colony and was decreased in use of α-GP only, were isolated, as the representative fosfomycin-resistant strains, and their characteristics were investigated.

1) Growth.

When NB was used, the growth rate of FR 90 was about 80 minutes in doubling time, while that of FR 95 was about 45 minutes (similar to the parental strain) as shown in Figs. 2 and 3.

Fig. 4. Utilization of carbohydrates and effect of c-AMP on the utilization in the resistant strains.
An overnight culture in NB was inoculated into M_9 medium containing glucose, glycerol, glycerol+c-AMP, α-GP or α-GP+c-AMP. The final concentration of carbohydrates was 0.4 % and c-AMP was 5 mM.



2) Carbohydrate utilization.

As shown in Fig. 4(a) and 4(c), FR 95 was decreased in utilization of α-GP but, when tested on glucose and glycerol, it grew at a rate indistinguishable from that of the parental strain. Further, it utilized normally the other carbohydrates described in Materials and Methods. On the other hand, FR 90 was incapable of growth on α-GP, glucose, or glycerol, as shown in Fig. 4(b) and the strain was defective in the utilization of the other carbohydrates except G-6-P. FR 90 could grow on G-6-P, but the onset of growth was delayed 3~4 hours in comparison with that of the parental strain (results not shown). Reason(s) of the delay is under examination.

3) Sensitization to fosfomycin.

KAHAN *et al.* reported that G-6-P potentiated fosfomycin on the drug-resistant cells shown to be *glp* T^- and its action was due to induction of a hexose phosphate transport system.[2] This potentiating effect on strains isolated by us was examined. Both FR 90 and FR 95 which were capable of growth in the presence of FOM at 200 μg/ml were sensitized to the drug by the addition of G-6-P to each growth medium, as shown in Fig. 2.

FR 90 was sensitized specifically by c-AMP, while FR 95 was not, as shown in Fig. 3. This effect was remarkable. In one case, FR 90 was sensitized to as little as 1 μg/ml of the drug. At the same time, c-AMP restored the utilization of α-GP and glycerol in FR 90 but the effect was not observed in FR 95, as shown in Fig. 4(b) and 4(c). Consequently, the sensitization of FR 90 by c-AMP is thought to result from "operation" of *glp* system, and therefore of the α-GP transport system, by the agent.[7]

From above-mentioned results, FR 90 phenotypically resembles a mutant which lacks enzyme I of the phosphoenolpyruvate phosphotransferase system (designated, *pts* I^-).[7,8] While, FR 95 seemed to be *glp* T^- which was reported by KAHAN *et al.*[2]

The fate of the fosfomycin-resistant strain of FR 90 type or FR 95 type, *in vivo*, and their virulence are under examination.

Acknowledgment

The authors are grateful to Prof. SABURO YAMAGISHI, Faculty of Pharmaceutical Science, Chiba University, and Dr. KOZO NAGAOKA, Pharmaceutical Development Research Laboratories, Meiji Seika Kaisha Ltd., for their helpful advices in the preparation of this manuscript.

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