

## Fosfomycin Inactivates Its Target Enzyme in *Escherichia coli* Cells Carrying a Fosfomycin Resistance Plasmid

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The target enzyme for fosfomycin antibiotic action, enolpyruvyl transferase, was synthesized in similar amounts and with similar kinetic and inhibition constants for phosphoenolpyruvate and fosfomycin, respectively, by an *Escherichia coli* strain containing the fosfomycin resistance plasmid pOU900 and by plasmid-free strains. Neither phosphoenolpyruvate level nor murein synthesis was altered by pOU900.

The antibiotic fosfomycin inactivates the enzyme phosphoenolpyruvate:UDP-*N*-acetylglucosamine-enolpyruvyl transferase (enolpyruvyl transferase; EC 2.5.1.7) by its structural analogy with phosphoenolpyruvate (PEP) (7, 8).

Fosfomycin-resistant mutants of *Escherichia coli* which fail to accumulate the drug are easily selected. The mutations are located in the chromosomal genes *glpT* and *uhp*, which control the transport of *L*- $\alpha$ -glycerophosphate and hexose phosphate, respectively (5, 10). Plasmids conferring fosfomycin resistance have recently been found in clinical isolates of *Serratia marcescens* (13). A 57-megadalton plasmid, pOU900, containing the fosfomycin resistance determinant on transposon Tn2921 (6) was transferred to the *E. coli* strain in which the mechanism of plasmid-mediated resistance was being investigated. The minimal inhibitory concentration of fosfomycin for *E. coli* 185(pOU900) is 24 mg/ml (9).

Transport experiments have demonstrated that *E. coli* 185(pOU900) takes up fosfomycin efficiently and accumulates it at concentrations high enough to inactivate the enolpyruvyl transferase from susceptible strains (9). Furthermore, when cells were transferred to medium without antibiotic, the intracellular fosfomycin in *E. coli* 185(pOU900), measured as described previously (9), did not show a significant decrease after 20 min of incubation, and no fosfomycin could be detected in the medium. These results rule out the active excretion of fosfomycin as the resistance mechanism. The aim of this work was to study how murein biosynthesis could occur under these conditions.

We first studied the fosfomycin target enzyme in plasmid-containing strains. The activity of enolpyruvyl transferase was determined in crude soluble extracts by measuring the incorporation of [<sup>14</sup>C]PEP (specific activity, 12.4 mCi/mmol) into charcoal-adsorbable material

was reported previously (15). The specific activity and  $V_{max}$  and  $K_m$  values for PEP, obtained from double reciprocal plots, were found to be similar for *E. coli* 185 and *E. coli* 185(pOU900)(Table 1). These results indicate that enolpyruvyl transferase from plasmid-containing strains is produced in similar amounts and has an affinity for the substrate similar to that produced by susceptible strains.

The sensitivity of the enzyme to fosfomycin was tested by preincubating the extracts in the presence of the drug and UDP-*N*-acetylglucosamine (which is required for enzyme inactivation [3, 8]) before the addition of PEP. Enolpyruvyl transferases from *E. coli* 185 and *E. coli* 185(pOU900) were inactivated to the same extent (Fig. 1). Moreover, both strains had similar  $K_i$  values for fosfomycin (Fig. 2). As  $K_i$  is related to  $K_m$  for each enzyme preparation, the  $K_m/K_i$  ratios were calculated and found to be about the same (Table 1). These results show that the target enzyme from cells containing pOU900 is fully sensitive to fosfomycin, despite their high resistance levels. Therefore, production of a resistant enzyme is not the plasmid-specified resistance mechanism.

The inducibility of fosfomycin resistance encoded by pOU900 was tested by growing *E. coli* 185(pOU900) cells in the presence of fosfomycin concentrations ranging from 5 to 200  $\mu$ g/ml and determining the minimal inhibitory concentrations as described previously (9). Although resistance was found to be noninducible, extracts from *E. coli* 185(pOU900) grown in the presence of fosfomycin (200  $\mu$ g/ml) were prepared and showed the same enzyme activity and  $K_i$  value for fosfomycin as those reported above.

Since the level of PEP available to the enzyme could be a significant variable, intracellular PEP levels were measured by endpoint enzymatic assay (2) in perchloric acid extracts of *E. coli* 185

TABLE 1. Properties of enolpyruvyl transferase from *E. coli* 185 and *E. coli* 185(pOU900) and PEP levels

<i>E. coli</i> strain	Sp act (nmol of PEP per min/mg of protein) <sup>a</sup>	$V_m$ (nmol of PEP per min/mg of protein)	$K_m$ ( $\mu$ M PEP)	$K_i$ ( $\mu$ M fosfomycin)	$K_m/K_i$ ratio	nmol of PEP per mg of cells (dry wt)
185	4.5	6.3	180	75	2.40	5.5
185(pOU900)	6	7	140	60	2.33	5

<sup>a</sup> Specific activity was measured under the assay conditions described in the legend to Fig. 1, but without preincubation with fosfomycin.

and *E. coli* 185(pOU900). However, no differences were found (Table 1), and the same level was found for *E. coli* 185(pOU900) grown in the presence of 200  $\mu$ g of fosfomycin per ml.

Another possible resistance mechanism is the determination by pOU900 of a murein biosynthesis pathway not requiring enolpyruvyl transferase. A different pathway might lead to differences in the overall synthesis rate and composition of murein. The rate was measured in *E. coli* W7 (a *dap lys* mutant) and its plasmid-containing derivative after the incorporation of *meso*-[3,4,5-<sup>3</sup>H]diaminopimelic acid (specific activity, 35 Ci/mmol) into murein sacculi as previously described (1). Murein was synthesised at the same rate (330 cpm) over 80 min in *E. coli* W7 and *E. coli* W7(pOU900). Murein cross-linkage was also determined and found to be the same in both strains as that previously reported for *E. coli* W7 (4).

If pOU900 could bypass the reaction catalyzed by enolpyruvyl transferase, it would probably generate an intermediate different from muramic acid after reduction of enolpyruvyl-UDP-*N*-acetylglucosamine. However, pOU900 did not complement the thermosensitive mutation carried by *E. coli* ST5, which has a defective enolpyruvyl-UDP-*N*-acetylglucosamine reductase (14). Complementation was assayed by monitoring growth of liquid cultures at permissive and restrictive temperatures. Since a bypass mechanism could involve other steps in murein synthesis, we also tested the ability of pOU900 to complement the mutations of H1119 (thermosensitive L-alanine adding enzyme [11]), PC1240 (thermosensitive diaminopimelic acid adding enzyme [12]), and PC1242 (thermosensitive D-alanyl-D-alanine adding enzyme [11]), but in none of these strains was the temperature-sensitive mutation suppressed, despite the conferrence of fosfomycin resistance.

The results presented here do not explain plasmid-coded fosfomycin resistance, but do shed some light on murein biosynthesis and the mode of action of the antibiotic. One explanation for our findings is that enolpyruvyl transferase is nonessential for murein biosynthesis and that another cellular function can provide this enzymatic step. This hypothesis would explain the lack of conditional mutants defective in this

enzyme. In addition, this hypothesis would imply that inactivation of enolpyruvyl transferase is not solely responsible for the antibiotic activity of fosfomycin.

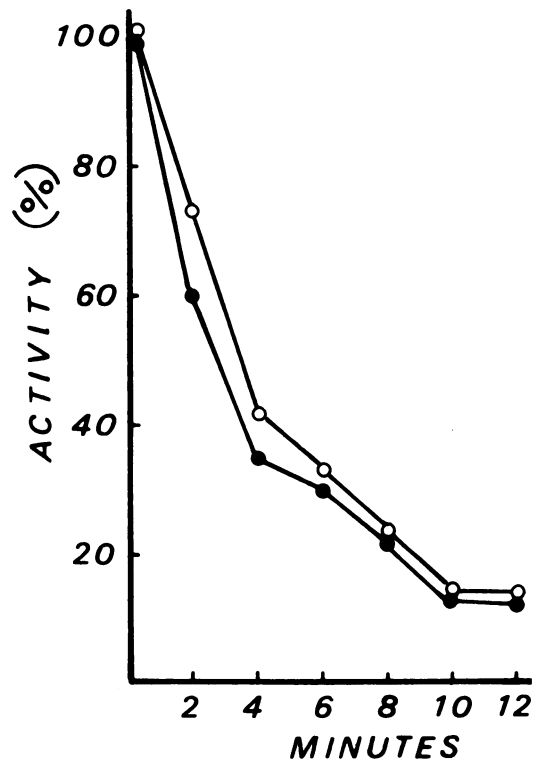


FIG. 1. Inhibition of enolpyruvyl transferase by preincubation with fosfomycin. Extracts were obtained by grinding bacteria grown to the stationary phase with alumina in the presence of 50 mM Tris-10 mM 2-mercaptoethanol (pH 7.5). After centrifugation at  $100,000 \times g$  for 90 min at 4°C, the supernatant was collected and used as the enzyme source. The reaction mixtures contained, in a final volume of 100  $\mu$ l, about 300  $\mu$ g of crude extract protein, 50  $\mu$ M fosfomycin, 50 mM Tris (pH 7.5), and 10 mM 2-mercaptoethanol. After various times, samples were placed into tubes containing [1-<sup>14</sup>C]PEP (final concentration, 0.5 mM), and the reactions were stopped after a further 20 min of incubation. ●, *E. coli* 185; ○, *E. coli* 185(pOU900).

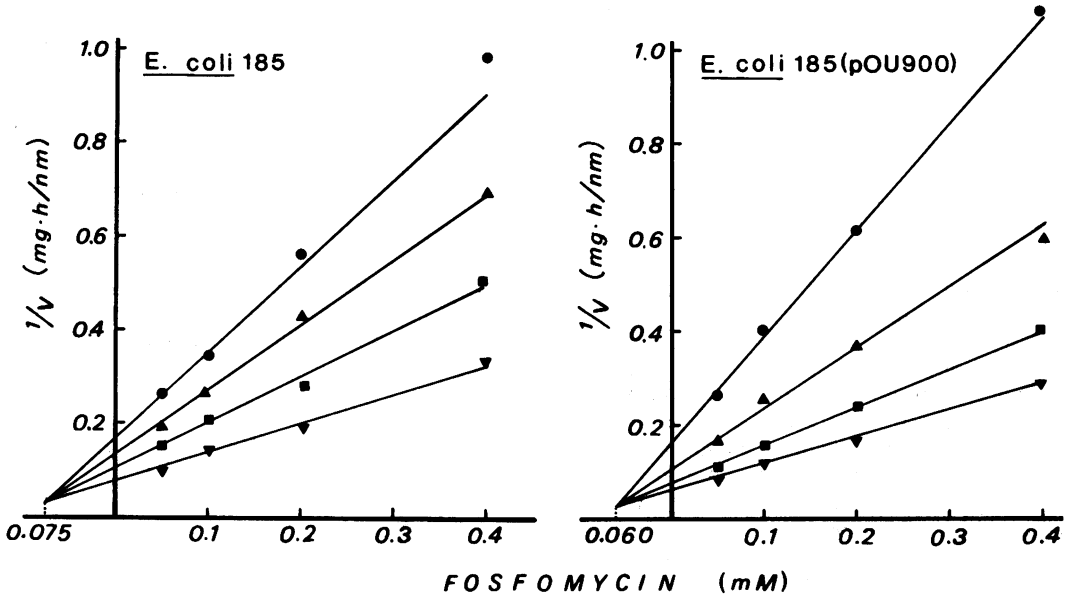


FIG. 2. Determination of apparent  $K_i$  values for fosfomycin of enolpyruvyl transferase from *E. coli* 185 and *E. coli* 185(pOU900). The reciprocal of initial velocities was plotted against the concentration of fosfomycin. The reactions were initiated by the addition of enzyme. Assay mixtures were the same as those described in the legend to Fig. 1, with the following concentrations of PEP: 0.25 mM (●), 0.50 mM (▲), 0.75 mM (■), 1 mM (▼).

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