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Plasmid-Mediated Fosfomycin Resistance Is Due to Enzymatic Modification of the Antibiotic

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The molecular mechanism of plasmid-mediated resistance to fosfomycin is described. The antibiotic was inactivated intracellularly and remained inside the cells. Modification was also obtained from cell extracts and was not energy dependent. The modifying enzyme seems to have sulfhydryl groups in its active center.

Fosfomycin is a small molecule (molecular weight, 138.1) with an unusual chemical structure, L-(*cis*)-1,2-epoxypropylphosphonic acid. Its inhibitory action is exerted on one of the first steps of peptidoglycan biosynthesis, namely, the condensation of uridine diphosphate-*N*-acetylglucosamine with phosphoenolpyruvate by means of a transferase which is actually the target site of the antibiotic (3, 4).

The antibiotic gains access to the bacteria by using the transport systems by which glycerol phosphate and glucose-6-phosphate normally penetrate bacteria (*glp*T and *uhp* systems, respectively) (4). Since these compounds are not essential for bacterial growth, chromosomal mutations which modify the expression of these permeases lead to fosfomycin resistance (4, 8).

In clinical strains isolated from members of the family *Enterobacteriaceae* collected in the Hospital N^a S^a Covadonga, Oviedo, Spain, we isolated plasmids which encode fosfomycin resistance. These plasmids have a high molecular weight, are conjugative, and carry other antibiotic resistance determinants (2, 7). They can be grouped into two categories by their incompatibility group, pattern of restriction, and DNA-DNA hybridization (2; unpublished results). They confer resistance to more than 1,024 μg of fosfomycin per ml, and their resistance determinants to this antibiotic are common to all of them (unpublished results). As has been reported, plasmid-mediated resistance cannot be explained by alterations in the drug transport systems (2, 5) or by modification of the target site (6). We tested the possibility that fosfomycin resistance was due to intra- or extracellular modification of the antibiotic by cells carrying plasmid resistance determinants. We used *Salmonella paratyphi* B 777 containing clinically isolated plasmids pUO900 or pUO500, representing the two groups indicated above, because in previous studies this strain showed the highest level of radioactive fosfomycin incorporation. *Escherichia coli* 803 containing the determinant of resistance to fosfomycin cloned into pBR322 (pUO001; C. J. Villar, J. E. Suarez, and C. Hardisson, submitted for publication) was also used because the increase in gene dosage could possibly increase its expression level. The organisms to be tested were grown to exponential phase in tryptic soy broth medium (Difco Laboratories) and then the cells were centrifuged and suspended in tryptic soy broth medium at a concentration of 200 mg (wet weight) of cells per ml. We added [^{3}H]fosfomycin (12.3 mCi/mmol, 100 μg/ml) to the culture and continued incubation at 37°C. At different time intervals, 500-μl portions were taken and centrifuged in an Eppendorf minifuge. The supernatant was kept, and the pellet was suspended in a mixture containing 100 μl of distilled water and 30 μl of toluene in 15% ethanol and thoroughly shaken for 10 min in a Vortex mixer and centrifuged. Samples of both the culture supernatant and the supernatant of the toluenized cells were then applied to Whatman 3MM paper and run for 16 h by descending chromatography with *n*-butanol–acetic acid–water (12:3:5) as an eluent. After drying, the chromatogram was cut into 1-cm strips, and the radioactivity of each was determined.

Figure 1a shows a chromatogram of pure [^{3}H]fosfomycin with the supernatant of toluenized fosfomycin-susceptible cells after 90 min of incubation in the presence of the drug. Only one peak of radioactivity is seen, indicating the radiochemical purity of the drug and that it is not modified by susceptible cells. However, when plasmid-containing strains were incubated with [^{3}H]fosfomycin, the supernatant of toluenized cells showed a peak of radioactive material migrating more slowly than that of [^{3}H]fosfomycin (Fig. 1b). In addition, no material corresponding to [^{3}H]fosfomycin was found in this extract, suggesting that the fosfomycin which penetrated the cells was modified. On the other hand, [^{3}H]fosfomycin was detected as the only radioactive material in the culture supernatant. As the time of incubation increased up to 90 min, the amount of slowly migrating material also increased, while the amount of [^{3}H]fosfomycin in the culture supernatant decreased. It is interesting that modified fosfomycin was never detected in the supernatant even after longer periods of incubation (4 h), which suggests that the modified fosfomycin remains inside the cell. The incubation of cells containing the multicopy plasmid pUO001 rendered approximately twice as much modified fosfomycin per time unit as that of cells harboring pUO900 or pUO500. The bioautography of the chromatogram with *Proteus vulgaris* MB838, a strain susceptible to 0.1 μg of the drug per ml, as a test organism and similar amounts of fosfomycin and its modified product (as judged by the number of radioactive counts) clearly indicated that modified fosfomycin was inactive as an antibiotic even in conditions under which fosfomycin gave inhibition halos of several centimeters.

A similar modification of fosfomycin, as tested by chromatography, was also found in vitro with cell extracts of *E. coli* 803 carrying plasmid pUO001 after centrifugation at 18,000 rpm for 4 h. The extracts were incubated with [^{3}H]fosfomycin (100 μg/ml) in 50 mM Tris-hydrochloride

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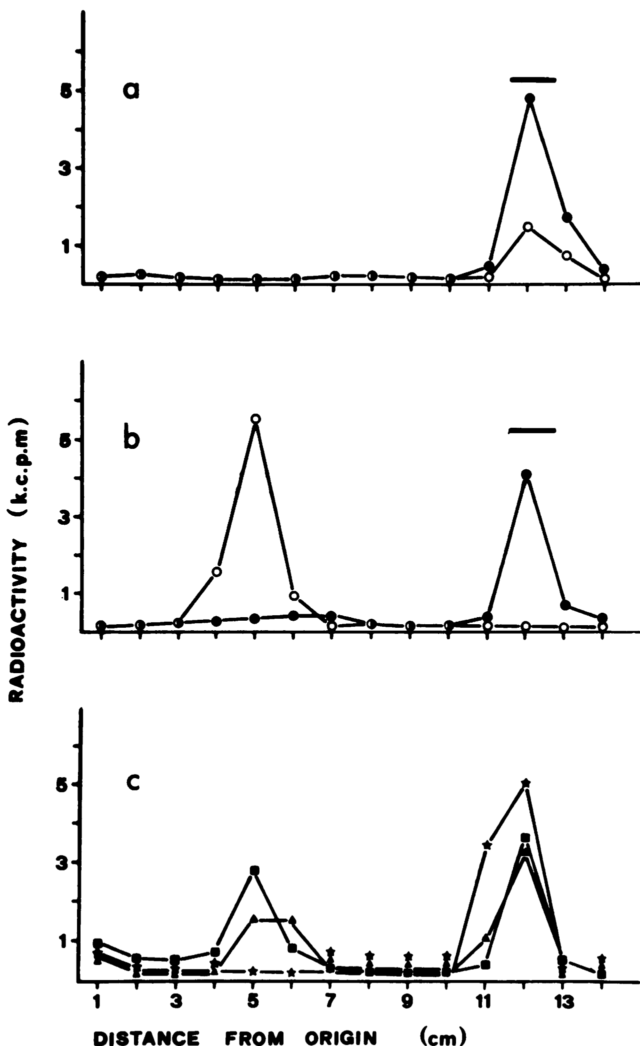

FIG. 1. Detection of modified fosfomycin by plasmid-mediated Fo^r cells. The separation of fosfomycin and its modified product was done by paper chromatography, as described in the text. The experiments were done with fosfomycin-susceptible cells (a), plasmid-borne cells (b), and plasmid-borne cell extracts (c). Symbols: ●, culture supernatants; ○, supernatants of toluenized cells; ■, cell extracts; ▲, cell extracts treated with 5 mM 2,4-dinitrophenol; ★, cell extracts treated with 1 mM *p*-chloromercuribenzoate. The horizontal bar corresponds to migration of pure [^{3}H]fosfomycin. In all the experiments shown the incubation time was 90 min.

buffer (pH 7.5). The inactivating capability was not affected in vitro by the presence of 5 mM 2,4-dinitrophenol but was strongly inhibited by 1 mM *p*-chloromercuribenzoate, proving that the enzyme does not require energy but contains reactive sulfhydryl groups in its active center (Fig. 1c).

The enzyme responsible for the modification of fosfomycin reported here has been recently identified as a 16,000- to 18,000-dalton polypeptide (1; Villar et al., submitted for publication) located in the cytoplasm of minicells containing the plasmid pUO001. This confirms our biochemical data of modification only inside the cells. In the near future we plan to study the nature of fosfomycin modification to understand the biological function of the resistance gene product.

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