Fosfomycin-resistance Plasmids Determine an Intracellular Modification of Fosfomycin

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Escherichia coli cells carrying fosfomycin-resistance plasmids modify fosfomycin intracellularly. The product of this modification (fosfomycin-derivative) differs from fosfomycin in chromatographic mobility, but the chemical nature of the modification is not yet known. Fosfomycin-derivative appears to have a cytoplasmic location and lacks antibiotic activity. The modification system can be saturated by an excess of fosfomycin in the incubation media.

INTRODUCTION

Fosfomycin (L-*cis*-1,2-epoxypropylphosphonic acid) is a broad-spectrum antibiotic produced by *Streptomyces* strains (Hendlin *et al.*, 1969) and is widely used in Spain and other countries. The bactericidal action of fosfomycin is due to the inactivation of the first enzyme of the murein biosynthetic pathway (phosphoenolpyruvate:UDP-*N*-acetylglucosamine enolpyruvyl-transferase, EC 2.5.1.7) by its structural analogy with phosphoenolpyruvate (Kahan *et al.*, 1974).

Plasmids conferring resistance to fosfomycin have been found in clinical isolates of *Serratia marcescens* (Mendoza *et al.*, 1980). A 57 MDal plasmid, pOU900, has been transferred to *Escherichia coli* strains to enable the mechanism of plasmid-mediated resistance to be studied. The resistance determinant of pOU900 is located on a transposon, Tn2921 (García-Lobo & Ortiz, 1982). A 1 kb DNA fragment from Tn2921 has recently been cloned into pBR322, and a plasmid-encoded polypeptide with a molecular weight of 16000 has been found to be responsible for the resistant phenotype (García-Lobo *et al.*, 1984).

Fosfomycin-resistant mutants of *E. coli* are easily obtained *in vitro*. Most of them show an impaired transport of the drug into the cell (Kahan *et al.*, 1974; Tsuruoka & Yamada, 1975; Dulaney & Ruby, 1977). However, previous work demonstrated that fosfomycin does enter plasmid-bearing cells (León *et al.*, 1982), and that fosfomycin inhibits the target enzyme of resistant cells *in vitro* (León *et al.*, 1983).

The aim of the investigation reported here was to elucidate how these plasmids determine the resistance to fosfomycin of the recipient bacteria.

METHODS

Bacterial strains, plasmids and growth conditions. *E. coli* C600 and *E. coli* 185 (resistant to nalidixic acid) are derivatives of *E. coli* K12 and are susceptible to fosfomycin. *E. coli* Gal5 and its derivatives 188 (*phoS*) and 207c (*phoS lky*) have been described elsewhere (Lazzaroni & Portalier, 1981). *Micrococcus varians* M11, a fosfomycin-hypersusceptible strain, was used as an indicator in drug bioassays. pOU900 is the original fosfomycin-resistance plasmid (Mendoza *et al.*, 1980). pSU912 is a ColE1 derivative carrying the transposon Tn2921 (García-Lobo & Ortiz, 1982). pSU902 is a pBR322 derivative containing the fosfomycin resistance gene from Tn2921 (García-Lobo *et al.*, 1984). In all the experiments, the bacterial cultures were grown in L broth (Davis *et al.*, 1980, p. 120) at 37 °C. Growth of cultures was measured at OD₆₀₀ with a Bausch & Lomb Spectronic 20 spectrophotometer.

Incubations with [³H] fosfomycin and preparation of cell extracts. In a typical experiment, bacteria were grown in 10 ml L broth to the end of exponential phase (OD₆₀₀ = 0.7). The cells were then centrifuged at 10000g for 10 min

in a Sorvall RC-5 centrifuge (20 °C) and washed with 60 ml L broth. The cells were finally resuspended in 2 ml L broth, and [^3H]fosfomycin (14.4 mCi mmol $^{-1}$; 533 MBq mmol $^{-1}$) was added to the final concentration specified for each experiment. After 20 min of incubation at 37 °C (unless otherwise stated) the cells were centrifuged in an Eppendorf 5414 centrifuge (5 min at room temperature) and washed twice with 10 mM-Tris/HCl buffer (pH 7.5). The cells were finally resuspended in 1 ml of the same buffer and ultrasonically disrupted (sonicator W-220F, Heat Systems Ultrasonics Inc., Plainview, NY, USA) by two pulses of 1 min at 4 °C. The sonicated samples were centrifuged in an Eppendorf centrifuge for 15 min at 4 °C and the supernates (crude extracts) were collected. When appropriate, the crude extracts were centrifuged at 100000 g (2 h at 4 °C) and the supernates (soluble extracts) were collected. Crude and soluble extracts were stored at -20 °C.

Paper chromatography. Samples of the extracts prepared as described above (10–40 μl , 200–1000 c.p.m.) were spotted on Whatman 3MM paper sheets (19 cm long) and subjected to ascending chromatography using 1-butanol/glacial acetic acid/water (12:3:5, by vol.) as the solvent. Once the solvent reached the end of the paper, usually after 6 h, the paper sheet was dried at room temperature and the strip corresponding to each sample (2 cm width) was cut into fractions of 0.5 $\times$ 2 cm. Samples containing 0.5 μg [^3H]fosfomycin were used as standards. The radioactivity of each fraction was determined by liquid scintillation counting using toluene-based scintillation fluid.

Bioassay of fosfomycin. Samples (5–20 μl) of the cell extracts and diluted culture broths were pipetted into wells on Mueller-Hinton agar plates (Difco) previously spread with a 10^{-4} dilution of an overnight culture of *Micrococcus varians* M11 (minimal inhibitory concentration 4 ng fosfomycin ml $^{-1}$; J. León, unpublished results). Bioassay of the chromatographic fractions was done by putting half fractions (0.5 $\times$ 1 cm) onto Mueller-Hinton agar plates inoculated as described above. This zones of inhibition of bacterial growth were measured and compared with those for standard fosfomycin solutions. This bioassay could detect as little as 2 ng of fosfomycin.

Other methods. Minimal inhibitory concentrations were determined by inoculating about 5×10^4 cells on Mueller-Hinton agar plates containing various concentrations of fosfomycin. Alkaline phosphatase activity was assayed with *p*-nitrophenyl phosphate as the substrate as previously described (Lazzaroni & Portalier, 1981). Protein was determined by the method of Bradford (1976) using bovine serum albumin as standard.

RESULTS

Fosfomycin modification by plasmid-carrying resistant cells

León *et al.* (1982) showed that plasmid-carrying strains accumulate fosfomycin after incubation in the presence of the drug. Extracts from *E. coli* 185(pOU900) incubated with [^3H]fosfomycin (50 μg ml $^{-1}$) were analysed by paper chromatography. The radioactivity appeared in the chromatogram in a different location from that of fosfomycin (Fig. 1a). Conversely, only [^3H]fosfomycin was found in the extracts of *E. coli* 185 (Fig. 1b). This means that fosfomycin was converted by plasmid-carrying cells into another compound with a different chromatographic mobility. This new compound, derived from fosfomycin, will be referred to in this paper as fosfomycin-derivative. The R_f of fosfomycin-derivative in this chromatographic system was 0.11 ± 0.022 (mean value from 12 independent experiments $\pm$ SD) while that of fosfomycin was 0.24 ± 0.035 .

The culture media from the experiments described above was analysed by paper chromatography and was found to contain no fosfomycin-derivative. However, the broth of the *E. coli* 185(pOU900) culture showed an antibiotic activity about 10% lower than that of the culture broth of the strain lacking the plasmid; this is in agreement with the amount of drug modified intracellularly by the resistant cells.

Intracellular fosfomycin modification was also assayed in *E. coli* C600 derivatives carrying either plasmid pSU912 or pSU902. These strains modified fosfomycin to the same extent as *E. coli* C600(pOU900); the modification does not depend on the recipient strain but on the fosfomycin-resistance gene from Tn2921. Unlike pOU900, pSU912 and pSU902 are multicopy plasmids, so the data reported above agree with previous results showing the lack of a gene dosage effect in the expression of the resistance to fosfomycin (García-Lobo *et al.*, 1984).

Fig. 2 shows the relative amounts of fosfomycin and fosfomycin-derivative found in crude extracts of *E. coli* 185(pOU900) after incubating the cells with increasing concentrations of [^3H]fosfomycin. All the radioactivity found in the extracts from cells incubated with low drug concentrations (50 μg ml $^{-1}$) corresponded to fosfomycin-derivative. When higher fosfomycin concentrations were used, increasing quantities of unmodified drug were found in the extracts.

Fig. 1. Paper chromatograms of extracts obtained from (a) *E. coli* 185(pOU900) and (b) *E. coli* 185. The cells were incubated for 20 min with $50 \mu\text{g } [^3\text{H}]\text{fosfomycin ml}^{-1}$. The arrows indicate the positions of fosfomycin markers. The origin was on fraction 1 and the solvent front on fraction 36.

Fig. 2. Effect of incubation with various concentrations of $[^3\text{H}]\text{fosfomycin}$ on the amounts of $[^3\text{H}]\text{fosfomycin-derivative}$ (□) and $[^3\text{H}]\text{fosfomycin}$ (▨) found in the extracts from *E. coli* 185(pOU900). The concentration of each compound in the extracts was determined by paper chromatography and expressed as c.p.m. (μg protein)⁻¹. Values inside the bars indicate the percentages of each compound with respect to the total radioactivity in the extract. The antibiotic activity of the extracts are expressed as ng fosfomycin (μg protein)⁻¹ (■). Results are mean values of two independent experiments.

The extent of fosfomycin modification also depended on the length of time of incubation in the presence of $[^3\text{H}]\text{fosfomycin}$. When the kinetics of intracellular modification of fosfomycin using low ($50 \mu\text{g ml}^{-1}$) and high (1 mg ml^{-1}) drug concentrations were determined, higher relative amounts of fosfomycin-derivative were found with shorter incubation times (Fig. 3).

Fosfomycin transport may be induced by glucose 6-phosphate (G6P) both in sensitive (Kahan *et al.*, 1974) and in plasmid-carrying strains (León *et al.*, 1982) by inducing the hexose-phosphate transport system (Dietz, 1976). The effect of this induction on the extent of fosfomycin modification was studied by comparing the extracts from cells grown in the absence or presence of G6P (5 mM) prior to incubation with $[^3\text{H}]\text{fosfomycin}$. Induction of fosfomycin transport resulted in higher modification (Table 1). Fosfomycin was also modified intracellularly by

Fig. 3

Fig. 3. Kinetics of fosfomycin modification by *E. coli* 185(pOU900) incubated in the presence of either 1 mg [^3H]fosfomycin ml^{-1} (○, ●) or 50 μg [^3H]fosfomycin ml^{-1} (□, ■). The extracts were analysed by paper chromatography, as described in Methods, to determine the amounts of [^3H]fosfomycin (○, □) and [^3H]fosfomycin-derivative (●, ■).

Fig. 4

Fig. 4. Antibiotic activity of fosfomycin-derivative. *E. coli* 185(pOU900) was incubated with [^3H]fosfomycin (1 mg ml^{-1}) for 20 min, and the cell extract was analysed by paper chromatography. Each paper fraction was divided into two halves. One half was used to measure the radioactivity (○) and the other one to measure the antibiotic activity (●), as described in Methods. The arrow indicates the position of a fosfomycin marker in the chromatogram.

Table 1. *Effect of the induction of fosfomycin transport by glucose 6-phosphate on the intracellular modification of fosfomycin by E. coli 185(pOU900)*

Cells were grown for 2 h in the presence or absence of 5 mM-G6P, washed with L broth and incubated with either 50 or 1000 μg [^3H]fosfomycin ml^{-1} as described in Methods. Results are mean values obtained from three experiments.

Treatment for induction	Concn of [^3H]fosfomycin ($\mu\text{g ml}^{-1}$)	Radioactivity in extract [c.p.m. ($\mu\text{g protein}^{-1}$)]	Amount of each compound in extract [c.p.m. ($\mu\text{g protein}^{-1}$)]*	
			Fosfomycin-derivative	Fosfomycin
None	50	25	25 (100)	0 (0)
5 mM-G6P	50	45	39 (86)	6 (14)
None	1000	59	34 (58)	25 (42)
5 mM-G6P	1000	84	41 (48)	43 (52)

* Values in parentheses are the relative percentages of each compound in the extract.

plasmid-carrying cells in the presence of 100 μg chloramphenicol ml^{-1} (data not shown); this is in agreement with previous results showing that fosfomycin resistance is non-inducible (León *et al.*, 1983).

Fosfomycin-derivative lacks antibiotic activity

In order to determine whether the fosfomycin-derivative showed antibiotic activity, the extracts obtained from *E. coli* 185(pOU900) cells incubated with various concentrations of [^3H]fosfomycin were assayed for antibiotic activity as described in Methods. Only the extracts that contained fosfomycin inhibited *Micrococcus varians* M11; the extracts that contained only fosfomycin-derivative did not (Fig. 2).

Table 2. *Effect of the ionic strength of the washing media on the concentrations of fosfomycin and fosfomycin-derivative in the extracts from E. coli 185(pOU900)*

Cells were incubated for 20 min with 0.5 mg [^3H]fosfomycin ml^{-1} , and were washed twice with distilled water or Tris buffer (pH 7.5) before sonication. Results are mean values of two independent experiments.

Tris concn in washing buffer (mM)	Total radioactivity in extract [c.p.m. ($\mu\text{g protein}^{-1}$)]	Amount of each compound in extract [c.p.m. ($\mu\text{g protein}^{-1}$)]*	
		Fosfomycin- derivative	Fosfomycin
0	44	39 (89)	5 (11)
10	42	35 (83)	7 (17)
50	60	44 (74)	16 (26)
100	66	36 (55)	30 (45)
150	70	35 (50)	35 (50)

* Values in parentheses are the relative percentages of each compound in the extract.

The antibiotic activity of chromatographic fractions was also tested. Crude extracts obtained from plasmid-carrying cells incubated with 1 mg [^3H]fosfomycin ml^{-1} were subjected to paper chromatography, and the paper fractions were bioassayed as described in Methods. The radioactivity of the fractions was also measured. Those fractions corresponding to fosfomycin-derivative lacked antibiotic activity (Fig. 4).

Intracellular location of the fosfomycin-derivative

As shown by the experiments reported above (Figs 2 and 3), both fosfomycin and fosfomycin-derivative were found in extracts of plasmid-carrying cells after incubation with high fosfomycin concentrations. However, when cells incubated in the presence of fosfomycin were washed with buffers of increasing Tris molarity, increasing amounts of unmodified fosfomycin appeared in the extracts, while the amount of fosfomycin-derivative remained unchanged (Table 2). This effect could be reproduced by using NaCl instead of Tris in the washing solutions (not shown). This result suggests a cytoplasmic location for fosfomycin-derivative, while a part of the fosfomycin would be in the periplasmic space. In another approach to determine the location of the fosfomycin-derivative, we transferred pOU900 to a periplasmic-leaky mutant, *E. coli* 207c (*phoS lky*), which releases periplasmic material into the extracellular medium (Lazzaroni & Portalier, 1981). The modification of fosfomycin was assayed after incubation with 1 mg ml^{-1} of the drug. The extent of this modification was compared with that mediated by pOU900 in the strain Gal5 (wild-type) and 188 (*phoS*, constitutive alkaline phosphatase). The results (Table 3) indicated that the *lky* mutation did not affect the amount of fosfomycin-derivative in the extracts, and, therefore, a periplasmic location for this compound is ruled out. Strain 207c(pOU900) showed a resistance level similar to that of parental strains. The leaky phenotype from 207c(pOU900) was confirmed by assaying alkaline phosphatase in the culture media as previously described (Lazzaroni & Portalier, 1981).

The former results suggested that fosfomycin-derivative was strongly retained in the cytoplasm of plasmid-carrying cells. To test this, *E. coli* 185(pOU900) incubated with [^3H]fosfomycin (1 mg ml^{-1}) for 20 min were put into a fosfomycin-free medium and incubated for a further 20 min. Analysis of the extracts by paper chromatography showed no significant decrease in the quantity of fosfomycin-derivative with respect to the control (data not shown); this compound did not permeate outside the cells.

When cell extracts containing fosfomycin-derivative were treated with 5% (w/v) trichloroacetic acid (30 min at 4 °C) and centrifuged, most fosfomycin-derivative (92%) was found in the supernate, indicating that it was not associated with macromolecules. Also, the fosfomycin-derivative in these extracts was dialysable.

Table 3. *Concentrations of fosfomycin and fosfomycin-derivative in the extracts from periplasmic-leaky mutants*

Cells were incubated for 20 min with 1 mg [^3H]fosfomycin ml^{-1} , and were processed as described in Methods except that they were washed three times before sonication. Results are mean values of two independent experiments, except for the minimal inhibitory concentrations.

<i>E. coli</i> strain	Relevant genotype	Amount of each compound in extract [c.p.m. ($\mu\text{g protein}^{-1}$)]*		Minimal inhibitory concentration (mg fosfomycin ml^{-1})
		Fosfomycin- derivative	Fosfomycin	
Gal5(pOU900)	Wild-type	46 (68)	21 (32)	40
188(pOU900)	<i>pho</i>	42 (63)	25 (37)	30
207c(pOU900)	<i>pho lky</i>	47 (82)	10 (18)	30
207c	<i>pho lky</i>	0 (0)	56 (100)	0.016

* Values in parentheses are the relative percentages of each compound in the extract.

DISCUSSION

The results reported here demonstrate that the plasmid gene specifying resistance to fosfomycin determines an intracellular chemical modification of the drug, generating a product with a different chromatographic mobility. This new product, named here fosfomycin-derivative, showed no antibiotic activity and was located in the bacterial cytoplasm (Tables 2 and 3). Fosfomycin-derivative may also be separated from fosfomycin by molecular filtration in Sephadex G-25 and electrophoresis on cellulose acetate (J. León, unpublished results), but its purification in amounts large enough to allow the elucidation of its structure is still to be accomplished.

The fosfomycin-modifying system could be saturated by an excess of drug; greater quantities of unmodified fosfomycin were detected in the extracts when higher concentrations of the drug were present in the incubations (Figs 2 and 3). However, most of the unmodified drug was released from the cells by lowering the ionic strength of the washing buffer (Table 2) or when a periplasmic-leaky mutant was used (Table 3), suggesting a periplasmic location for the unmodified fosfomycin. This is not an unexpected result as fosfomycin, in the absence of G6P, enters the bacteria through the glycerol 3-phosphate (G3P) transport system *glpT* (Kahan *et al.*, 1974; León *et al.*, 1982). A periplasmic component is involved in G3P transport, and the release of G3P from *E. coli* cells by water or low ionic strength media is a well-documented phenomenon (Hayashi *et al.*, 1964; Silhavy *et al.*, 1976).

In our previous work we could not detect intracellular modification of fosfomycin by plasmid-bearing strains (León *et al.*, 1982). This was due to the experimental conditions that were used (incubations with 2 mg fosfomycin ml^{-1} for 40 min and hypertonic buffer used for washing the cells), which favoured the presence of high levels of unmodified fosfomycin in the extracts. Also, the extracts were bioassayed using an indicator strain (*Staphylococcus aureus* ATCC 25923) which is about 4000 times more resistant to fosfomycin than is *Micrococcus varians* M11. Consequently, the bioassays reported in the present paper are much more sensitive.

When transport of fosfomycin into plasmid-carrying cells was induced with G6P, higher amounts of fosfomycin-derivative were found in the extracts (Table 1). This indicates that the modifying enzyme is cytoplasmic, since it modifies fosfomycin previously transported to the bacterial cytoplasm. As noted above, we have previously identified a Tn2921-coded polypeptide (molecular weight 16000) associated with fosfomycin resistance. This FOS polypeptide seems likely to correspond to the enzyme which catalyses the modification of fosfomycin. The FOS polypeptide has been found in the cytoplasmic fraction of plasmid-carrying *E. coli* minicells (unpublished results), which is in agreement with the result discussed above. However, there is not yet direct evidence of the intracellular location of the modifying enzyme, as experiments with the aim of reproducing fosfomycin modification *in vitro* have so far failed. This failure may

be due to the omission of some cofactor required for the modification reaction. Elucidation of the structure of fosfomycin-derivative and establishing the conditions required for the modification reaction *in vitro* should allow the study of the plasmid-coded fosfomycin-modifying enzyme.

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