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MINIREVIEW

Plasmid-Encoded Fosfomycin Resistance

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Fosfomycin [(−)-cis-1,2-epoxypropyl phosphonic acid] was discovered in 1968 as a secondary metabolite of several *Streptomyces* species (28). It aroused considerable interest, since it was a completely new molecule having antibacterial activity which exhibited relevant properties, such as a broad spectrum, covering many pathogenic and opportunistic gram-positive (*Staphylococcus aureus* and *Streptococcus* spp.) and gram-negative (*Pseudomonas aeruginosa*, *Neisseria* spp., and members of the family *Enterobacteriaceae*) bacteria (5, 19, 35, 43, 68). Furthermore, in combination with other antimicrobial agents, it was synergistic (17, 24, 50, 51, 61). Its chemical structure (Fig. 1) (12) presented new features, with a stable epoxide group and a direct carbon-phosphorus bond, both of which were necessary for therapeutic activity (12, 34); these features explained the absence of cross-resistance with other antibiotics used in therapy (55). Fosfomycin, acting as a phosphoenolpyruvate analog, irreversibly inhibited phosphoenolpyruvate UDP-*N*-acetylglucosamine-3-*O*-enolpyruvyl transferase (enolpyruvyl transferase) (11, 34, 53, 54, 65), an enzyme which catalyzed the first step of peptidoglycan biosynthesis. It has almost no toxicity in humans (18, 28). It does not have an affinity for plasma proteins, in part explaining its lack of induction of allergic reactions (35, 55). Fosfomycin can be administered both parenterally (disodium salt) and orally (calcium, lysine and, more recently, trimethamine salts) (9, 10, 16, 21, 26) and shows excellent penetration into body fluids (15, 21, 52), with renal excretion of the active compound and short clearance times (9, 21, 35). Finally, it is taken up by eucaryotic cells and acts against intracellular parasites (29).

Fosfomycin is taken into cells by active transport through the partially constitutive L-α-glycerophosphate uptake system (GlpT), as demonstrated by the finding that GlpT mutants are resistant to fosfomycin (Fo^r) and that constitutive mutants appear to be hypersusceptible to the drug (34). A secondary transport system, which mediates hexose phosphate uptake (Uhp), can be induced by glucose 6-phosphate. Under such conditions, transport of and susceptibility to fosfomycin by GlpT$^-$ mutants became comparable to those of wild-type strains (34). Most chromosomally resistant mutants have an impairment in one or both of these uptake systems (33, 34, 60). Mutations that diminish the affinity of fosfomycin for its target enzyme also make cells resistant, although in this case there is a concomitant increase in the K_m for phosphoenolpyruvate, resulting in a lower rate of synthesis of peptidoglycan (62).

The use of fosfomycin has selected another type of resistant organisms which actively incorporate the drug (27, 37, 45) and have a fully sensitive enolpyruvyl transferase (38), indicating that their mechanism of resistance is different from that of the previously described mutants. They are usually multiresistant, can transfer their resistance through conjugation or transformation, and can be made susceptible through the use of curing agents, indicating a plasmid location for the Fo^r determinant (27, 44, 49). These strains were discovered as a consequence of a screening for Fo^r among *P. aeruginosa* and *Serratia marcescens* isolated in our hospital, done to evaluate the prospects of use of the antibiotic. All *P. aeruginosa* strains were transport mutants, but several *S. marcescens* strains actively incorporated radioactive fosfomycin and transferred Fo^r, together with other resistances, to *Escherichia coli* through conjugation (45, 60). Plasmid analysis of the transconjugants showed that a majority contained an 88-kbp plasmid (pUO900), later classified as IncM (64), which conferred resistance to ampicillin and streptomycin-spectinomycin as well. The rest harbored a ca. 170-kbp IncC plasmid which carried resistances to ampicillin, chloramphenicol, sulfonamides, tetracycline, and several aminoglycosides (pUO500) (27, 45). Further screenings of strains isolated in our hospital and community revealed a slow but continuous increase in both the frequency and the number of species carrying the Fo^r determinant. Thus, conjugative plasmid-encoded Fo^r has extended to *Serratia liquefaciens* and *Klebsiella oxytoca* (IncM plasmids of 85 to 110 kbp) and to *K. pneumoniae*, *K. oxytoca*, and *E. coli* (IncC plasmids of 100 to 185 kbp) (27, 44). No transmissibility was found among *Enterobacter cloacae*, *Proteus* and *Morganella* spp., *Providencia stuartii*, and *Pseudomonas* spp. However, 75% of Fo^r enterobacteria and 50% of Fo^r *Pseudomonas* spp. were capable of growth in L-α-glycerophosphate, possibly indicating either that the lack of Fo^r transmission was a consequence of the presence of the determinant in a nonconjugative plasmid or in the chromosome or that the receptors of the matings were not appropriate. At present, Fo^r frequency seems to have reached an equilibrium in our hospital, as judged by the results of yearly screenings, since 1982, of *S. marcescens* isolates from a variety of pathological samples, which show around 50% susceptibility, with a slight tendency toward lowering (46). Furthermore, screenings of blood and urine samples consistently show more than 90% susceptibility of *E. coli* and *S. aureus*, between 80 and 45% susceptibility of *Staphylococcus epidermidis*, between 50 and 35% susceptibility of *P. aeruginosa*, and between 50 and 66% susceptibility of *S. marcescens*. A similar steady state seems to be the situation in La Fe Hospital in Valencia, Spain, the only other place in which, to our knowledge, regular studies of this type have been performed. In this case, comparisons of the frequencies of susceptibility to fosfomycin during the period from 1973 to 1984 have revealed consistent 90% susceptibility of *S. aureus*, *Salmonella* spp., *Shigella* spp.,

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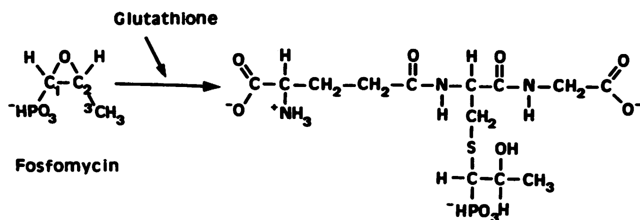

FIG. 1. Fosfomycin-modifying reaction (3).

and *E. coli*; 80 to 90% susceptibility of *Streptococcus faecalis*, *Klebsiella* spp., *Serratia* spp., and *Proteus mirabilis*; and over 70% susceptibility of *Enterobacter* spp. and *P. aeruginosa* (25). The overall susceptibility frequency in Valencia is 82%, and the frequencies, considered on a species basis, are somewhat more favorable in Valencia than in Oviedo. Significantly, no plasmid-encoded Fo^r could be found in a sample of Fo^r strains isolated in Valencia, on the basis of their hybridization with an 863-bp DNA segment which contains the Fo^r determinant and which was determined to be specific (see below). The last screening done in our hospital indicated that more than 70% of strains showing the phenotype Fo^r hybridized with the probe and that, among these strains, all modified fosfomycin (see below). The gene responsible for plasmid-encoded Fo^r has been detected in strains isolated in a hospital in Madrid but not in hospitals in other Spanish regions, indicating a certain degree of endemism (58).

To determine whether the plasmid-encoded Fo^r character was being spread from the hospital to the environment, we analyzed samples of sewage from residual waters from rural and urban communities, including sewage from the hospital. There were few Fo^r strains (2×10^{-6}) and, among these strains, the presence of conjugative Fo^r plasmids could be demonstrated in *K. oxytoca*, *E. cloacae*, *Proteus vulgaris*, and *Citrobacter freundii*. These plasmids, with sizes of 57 to 70 kbp, were classified as IncM; their DNA hybridized with the probe cited above, and extracts from the corresponding cells modified fosfomycin, confirming the identity of these strains with the hospital strains (1). Analysis of Fo^r isolates from sewage samples taken from seven plants scattered over Spain has shown positive hybridization and modification by enterobacteria of several species at all but one of the plants, which amounted to about 28% of the Fo^r isolates; by *Pseudomonas* spp. (5%) (at three locations); and, most surprisingly, by three colonies of *Staphylococcus xylosus* (58).

The Fo^r determinant was cloned into the *Bam*HI site of pBR322 and localized to an 863-bp segment flanked by *Bgl*II and *Pst*I sites (56, 63). Analysis of the sequence indicated that only one complete open reading frame (ORF), which was 432 nucleotides long and encoded a 15,941-dalton polypeptide, was included in it (56). Confirmation that this ORF corresponded to the fosfomycin gene (*fosA*) was derived from transposon mutagenesis with the γδ sequence and from the subclones generated to sequence the fragment; in every case, insertions of γδ or deletions affecting the ORF resulted in fosfomycin susceptibility (56, 63). Furthermore, minicell analysis of the polypeptides made by different Fo^r plasmids revealed a 16-kDa polypeptide which resulted from their expression and which was absent when γδ was inserted into the ORF (22, 63). Finally, the amino-terminal end of a protein capable of fosfomycin modification (see below) corresponded to the 10 first codons of the ORF (3). Expression of the cloned gene was constitutive, as judged by the intensity of the bands produced by minicell-producing strains grown and labeled in the presence or absence of the antibiotic (63). The gene is located in an 11.8-kbp transposon which does not carry other resistances or other selectable traits, to our knowledge. This transposon, obtained from pUO900 and called Tn*2921*, was demonstrated to be able to transpose successively from a clinical plasmid into ColE1 and RP4 in the absence of the RecA function (23). It is flanked by directly repeated sequences which hybridize to the insertion sequence IS*10* (48).

Cytoplasmic extracts of cells that harbor Fo^r plasmids and are grown in the presence of radioactive antibiotic contain a radioactive compound which does not comigrate with fosfomycin in chromatograms and which lacks antibiotic activity, indicating that fosfomycin is inactivated upon entry into resistant cells (36, 39). The intracytoplasmic location of the Fo^r protein is confirmed by the absence of inactivated fosfomycin in culture supernatants of cells containing Fo^r plasmids and by the presence of the 16-kDa polypeptide in the cytoplasmic fraction of minicells obtained from Fo^r cells (63). Furthermore, no discernible leader peptides or transmembrane helices are present in the protein, as deduced from its DNA sequence (3, 56).

The isolation of modified fosfomycin from the cytoplasm and the characterization of this compound through nuclear magnetic resonance studies showed that fosfomycin is converted in vivo to a glutathionyl derivative, via the opening of its epoxide group and the formation of an adduct between carbon atom 1 and the sulfhydryl group of the cysteine of the tripeptide glutathione (Fig. 1) (4). The role of glutathione in fosfomycin modification was further confirmed by the lack of resistance shown by cells containing Fo^r plasmids but unable to synthesize the tripeptide and also by the direct relationship between the degree of fosfomycin modification and the amount of exogenous glutathione added to extracts of Fo^r cells. Adduct formation appeared to be specific for glutathione: none of other several thiols tested could modify fosfomycin (4). The enzymatic activity of the Fo^r protein superficially resembles those of glutathione *S*-transferases (GST), which are ubiquitous in eucaryotic organisms. Furthermore, glutathione is an obligate substrate for those enzymes and is used to bind harmful compounds (41), thus protecting the cells in a similar way. However, there are differences between them: eucaryotic GST cannot modify fosfomycin (4) and, conversely, the bacterial enzyme does not support their typical reaction of binding between glutathione and 1-chloro-2,4-dinitrobenzene (3). Furthermore, there is no detectable homology between either kind of enzyme at the level of DNA or protein sequences. Finally, eucaryotic GST have secondary and tertiary structures of type α/β, while the bacterial enzyme is predicted to be an antiparallel α protein, possibly containing a divalent cation as a cofactor (3). (Recently, several reports on the isolation of "more typical" procaryotic GST have appeared, although no real function has been ascribed to them [2, 13, 31].)

The fosfomycin-modifying enzyme has been purified and shown to be a dimer of two 16,000-dalton polypeptides (the monomer has no enzymatic activity) which is able to support the reaction in the sole presence of the two substrates, fosfomycin and glutathione, although its activity is enhanced by the presence of Fe^{2+}, Co^{2+}, and Mn^{2+} ions and reduced by EDTA. Other conditions that optimally favor the reaction are a pH of 8.25 and temperatures of 20 to 30°C. On the other hand, phosphoenolpyruvate does not interfere with the fosfomycin modification reaction (3). Surprisingly, the K_m of

fosfomycin is 9.4 mM, a value which is about 100 times higher than that of the antibiotic for its target enzyme, enolpyruvyl transferase (38). However, cells containing the fosfomycin-modifying enzyme are resistant, presumably because the conditions of the reaction, in which purified enzyme and substrates are used, are not comparable to those of the cells. It is also possible that during the purification, an enhancer or cofactor is eliminated, resulting in a lower affinity of the protein for its substrates. On the other hand, the amounts of Fo^r protein made by minicells harboring pBR322 with *fosA* cloned in it almost equal those of β-lactamase (63), suggesting that resistance is due, at least partially, to the high intracellular concentration of the Fo^r protein. (The MIC of fosfomycin for cells containing single-copy plasmids is 4 mg/ml, while in those containing multicopy plasmids, the MIC is at least 20 mg/ml, the difference being due presumably to larger amounts of the protein being made as a consequence of increased gene dosage.)

The 863-bp DNA segment containing *fosA* was found to hybridize with total DNA of *Streptomyces fradiae* ATCC 21096, *S. viridochromogenes* ATCC 21240, and *S. wedmorensis* ATCC 21239, originally described as producers of fosfomycin. This result suggests the existence of sequences related to *fosA* in the DNA of these organisms and probably that these sequences all share a common origin. This origin would probably be *Streptomyces* because (i) the homology is not restricted to the fosfomycin producers but extends to others which have not been described as such, indicating that the *fosA* sequence has a wide distribution in the genus (56); (ii) structural analysis of *fosA* reveals features characteristic of *Streptomyces* genes; and (iii) the optimal temperature of the fosfomycin modification reaction, between 20 and 30°C (3), is more typical of a soil organism, adapted to frequent changes in environmental conditions, than of a human parasite, well adapted to the internal conditions of the body. Streptomycetes have a characteristically high G+C content, forcing a nonrandom use of triplets which are rich in G and C. As the degeneracy of the genetic code mainly occurs through changes in the third nucleotide of each triplet, there is an accumulation of G and C in that position. Consequently, it has been possible to develop a method to locate *Streptomyces* genes simply by measuring the G+C percentages in the nucleotides in the first (moderately high), second (relatively low), and third (extremely high) positions of the possible triplets of a sequence (8). The *fosA* gene resembles *Streptomyces* genes because, of the 61 coding triplets, 18 are not used at all, and 17 of these have an A or a T in the third position. Furthermore, the gene has a moderately high G+C content (67.4%) in the first position of each triplet, a relatively low content (47.8%) in the second, and a very high content (85%) in the third (56). Concurrently, at 7 nucleotides before the start of translation there is a sequence of 6 nucleotides which is exactly complementary to the 3′ end of *Streptomyces lividans* 16S rRNA (7). A streptomycete origin of the Fo^r sequence would be a new confirmation, at the DNA level, of a theory, first proposed by Walker and Walker (66) and later extended by Benveniste and Davies (6), that the genes of antibiotic resistance present in pathogenic bacteria originated in the genomes of the producers, which needed them as a means of escaping the toxicity of the antibiotics. Other cases in which this origin can be traced are an aminoglycoside-3′-fosfotransferase (59) and an aminoglycoside-3-*N*-acetyltransferase (40). In fact, it is now clear that proteins capable of molecular modification of antibiotics are included in the biosynthetic pathways of several antibiotics, where they may even regulate the expression of the biosynthetic genes (for a comprehensive review, see reference 30).

Recently, another plasmid-encoded Fo^r determinant (*fosB*) was found in a plasmid of *S. epidermidis* (14). This new gene encodes a 139-amino-acid protein with a molecular weight of 16,345. Although no hybridization with *fosA* could be observed, sequence analysis revealed 35% identity (nucleotides) and 38% identity (amino acids) with the gene isolated from *Serratia* plasmids. The latter value becomes 47.5% when only nonconservative changes of amino acids are considered. The level of correspondence, together with similar hydropathy profiles and the fact that changes are scattered throughout the proteins, suggests that *fosB* has the same origin as *fosA* (67). Further confirmation could come from the study of the effect of *fosB* on fosfomycin.

It seems that plasmid-encoded Fo^r is spreading both geographically (isolates have been obtained in different parts of Spain and in France) and biologically (the genes now seem to be present in gram-positive and -negative pathogenic bacteria), reflecting the clinical use of fosfomycin. However, it appears that the genes may have difficulty in colonizing hospital environments, even those in which fosfomycin is commonly used. This difficulty should prolong the usefulness of fosfomycin, both as a single agent and, possibly more importantly, in combination with other antibiotics, most promising being its combination with β-lactams and other peptidoglycan synthesis inhibitors (24, 50, 51, 61), quinolones (17), erythromycin (50), and aminoglycosides, with the added bonus in the last case of protection against glomerular damage (20, 32, 47), an effect that seems to be quite general, as fosfomycin also protects against renal damage induced by vancomycin (42) and the antitumor drug cisplatin (57). Another potentially interesting combination could be with glucose 6-phosphate, which has been shown to greatly enhance the therapeutic efficacy of fosfomycin in mice (34).

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