Fosfomycin Resistance in *Escherichia coli* in Japan

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Fosfomycin, [L-(*cis*)-1,2-epoxypropyl phosphonic acid] (FOM), a broad spectrum antibiotic produced by some strains of *Streptomyces* species[1], acts as a phosphoenolpyruvate analog and irreversibly inhibits phosphoenolpyruvate UDP-*N*-acetylglucosamine (UDP-GlcNAc)-3-*O*-enolpyruvyl transferase, an enzyme which catalyzes the first step of peptidoglycan biosynthesis[2]. FOM enters susceptible bacteria by means of two different transport systems[3~5]. The first, the GlpT system, transports L-α-glycerophosphate and can be potentiated under anaerobic conditions[5]. The second, the Uhp system, transports hexose phosphate and can be induced by glucose 6-phosphate (G6P)[4]. It has been reported that there are two types of FOM resistance, mediated by plasmid or chromosomal genes. Plasmid-encoded FOM resistance found in *Serratia marcescens* and *Staphylococcus epidermidis* is due to the *fosA* and *fosB* genes, respectively[6,7], encoding an enzyme FOM: glutathione *S*-transferase. FOM resistant *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* also produce FOM: glutathione *S*-transferase[8,9]. The enzymatic properties of FOM: glutathione *S*-transferase superficially resemble those of the glutathione *S*-transferases[10], which are ubiquitous in eucaryotic organisms. On the other hand, it has been reported that chromosomally-encoded FOM-resistance leads to a reduction in uptake of FOM by the cell[11], and is related to a defective in the L-α-glycerophosphate transport system (*glpT* gene)[12], a mutant which lacks enzyme I of the phosphoenol pyruvate phosphotransferase system (*ptsI* gene)[13] and others[12]. The MICs of FOM against clinical isolates of *Escherichia coli* has been described in many reports but there are few papers dealing with the mechanism of FOM resistance[14]. The present study was performed to determine FOM MICs for *E. coli* clinical isolates in Japan in 1996 and to analyze the mechanisms underlying development of FOM resistance.

Materials and Methods

Four hundred and forty-nine strains of *E. coli* clinical isolates obtained throughout Japan obtained at random in 1996 were studied. *E. coli* K-12 W3110 Rif^r was used as a recipient in transconjugation experiments to identify the presence of R plasmids.

Sodium fosfomycin (FOM) and [2,3-^{3}H]fosfomycin ([^{3}H]FOM: 37 MBq/3.4 mg) were obtained from Meiji Seika Kaisha, Ltd., Tokyo, Japan. Nutrient broth (Eiken Chemical Co., Ltd., Tokyo, Japan), nutrient agar (Difco Laboratories, Inc., Detroit, MI, USA) and L-broth described by LENNOX *et al.*[15] were used for bacterial culture and other components were all commercially supplied.

MICs were determined by inoculating 10^{-3}-fold diluted cells of overnight cultures on nutrient agar plates containing FOM in two-fold dilutions starting from 12,800 μg/ml to 0.39 μg/ml[16]. After incubation for 18 hours, MICs for each strain was determined.

The capacity for transfer of FOM resistance was determined by modifying the broth mating method[8,17]. Briefly, 0.1 ml of a nutrient broth culture in late log growth phase of donor strains and 0.9 ml of the recipient strain *E. coli* W3110 Rif^r were mixed with 9 ml of fresh nutrient broth. Frequency of transfer was measured by selection on nutrient agar plates containing 200 μg/ml of FOM and 100 μg/ml of rifampicin after mating for 2 hours.

For the preparation of crude extracts, exponentially growing cells were harvested by centrifugation at 10,000 × *g* for 20 minutes and washed with 0.85% NaCl. Cells from 10 ml culture were suspended in 1 ml of TMK solution, consisting of 60 mM KCl, 10 mM $MgCl_2$, 6 mM 2-mercaptoethanol and 100 mM Tris-HCl buffer (pH 7.8), and disintegrated by sonic oscillation (Branson Sonifier Cell disrupter Model W-200P, USA) for 0.5 minutes, 4 times. The supernatant fluid (crude extract) was obtained by centrifugation at 20,000 × *g* for 30 minutes at 4°C.

To test for drug inactivation, reaction mixtures containing 140 μl of crude extract, 25 μl of Co-factors (80 mM glutathione, 40 mM ATP or 2 mM acetyl Co A), 25 μl of FOM (31.3 μg/ml) and 60 μl of TMK solution was incubated at 37°C for 18 hours. A 20 μl portion of the reaction mixture was spotted on paper discs (Toyo

Thin Disk, 8 mm dia Thin, TOYO Filter Co. Ltd. Tokyo, Japan). After heating twice in a microwave oven (Toshiba Microwave Oven Model ER-613S, Tokyo, Japan) for 15 second each, the residual activity of FOM in the discs was determined by microbiological assay.

The uptake of FOM was determined under different culture conditions. Effects of G6P were determined by inoculating 10^{-3}-fold diluted cells from strain M49 or M68 of overnight cultures on nutrient agar plates containing 50 μg/ml of G6P. Anaerobic conditions were established with AeroPack·KENKI (Mitsubishi Gas Chemical Co. LTD., Tokyo, Japan).

The incorporation of [^{3}H]FOM into cells was determined as follows. An overnight L-broth culture was diluted 2×10^{-2}-fold and incubated for about 90 minutes at 37°C with shaking to an OD of 0.3 at 600 nm. At this point, G6P was added to a final concentration of 5 mM, and the culture was incubated for 90 minutes. Bacterial cells were centrifuged at $6{,}000 \times g$ for 15 minutes, washed with L-broth, resuspended in 2 ml of L-broth and mixed with 0.02 mM [^{3}H]FOM and 0.62 mM FOM. Periodically, an 0.3 ml sample was removed and filtered through a membrane filter (0.45 μm pore size), which was washed with 10 ml of hypertonic buffer consisting of 0.5 mM $MgCl_2$, 150 mM NaCl and 10 mM Tris-HCl (pH 7.0)[8], and the radioactivity on the filter was determined in an Aloka LSC-5100 liquid scintillation counter in a toluene-based fluid.

Results and Discussion

The distribution of FOM MICs in 449 strains of *E. coli* clinical isolates obtained in Japan in 1996 exhibited a sharp peak at 12.5 μg/ml; 99.8% of the clinical isolates tested were susceptible to FOM (data not shown). Only one FOM-resistant strain M49 demonstrated an MIC value of 800 μg/ml. Goto *et al.*[16] reported MIC_{50} and MIC_{80} values of 6.25 μg/ml and 12.5 μg/ml for clinical isolates of *E. coli* in 1975. The MIC_{80} range of clinical isolates used in the present study remained unchanged when compared with the 1975 report. The proportion of FOM-resistant strains among clinical isolates of *E. coli* has not changed in Japan since 1980, when FOM was first introduced for clinical application.

The frequency of transfer of FOM resistance from the FOM-resistant strain M49 to a susceptible strain W3110Rif^{r} was less than 10^{-8} (data not shown). While Suárez *et al.*[6] and Etienne *et al.*[7] have reported that plasmid-encoded FOM-resistance has disseminated in Spain, reflecting the widespread clinical use of FOM,

Table 1. Effects of culture conditions.

	MIC (μg / ml)			
	Aerobic		Anaerobic	
Strain	G6P-	G6P+	G6P-	G6P+
E. coli M68	12.5	6.25	6.25	6.25
E. coli M49	800	800	200	200

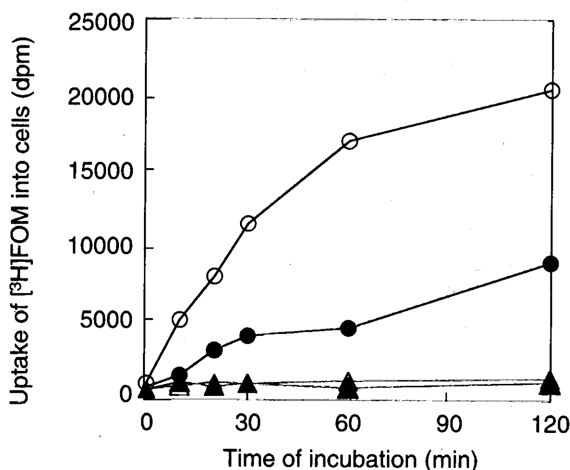
Arca *et al.*[18] reported that plasmid-determined FOM resistance accounts for only a low percentage of the FOM resistant strains in Italy. Such does not appear in Japan and the present study showed lack of transfer of FOM resistance from strain M49. In an *in vitro* study, Baquero *et al.*[19] found that in 65% of 60 FOM resistant strains of Gram-negative bacilli including 4 *E. coli* isolates, plasmid-determined resistance to other antimicrobials was transferred to *E. coli* K-12, but was not accompanied by demonstrable FOM resistance. The apparent difficulty in transferring the FOM resistance gene should prolong the useful life of FOM both as a-single agent and, possibly more importantly, in its combination with other antibiotics. In this regard, β-lactams and other peptidoglycan synthesis inhibitors as well as aminoglycosides are promising[6,20~22]. The result of our transfer study of FOM resistance suggest that plasmid-encoded FOM resistance is rare at present in clinical isolates of *E. coli* in Japan, and that plasmid-encoded FOM resistance in clinical isolates of *E. coli* has not yet spread in Japan.

A crude extract from strain M49 did not inactivate FOM in the presence of glutathione, although that from FOM: gluthathione *S*-transferase-producing *P. aeruginosa* T-75[9] caused complete loss of the activity (data not shown). The role of glutathione in FOM modification by glutathione *S*-transferase was confirmed by a decrease in antibiotic activity of crude extracts from strains containing a FOM-resistance plasmid[10]. In this study, we were unable to demonstrate the inactivation of FOM in the FOM-resistant strain M49.

Under aerobic conditions, the antimicrobial activity of FOM against susceptible strain M68 was enhanced from 12.5 μg/ml to 6.25 μg/ml in the presence of G6P while the activity of FOM against the resistant strain M49 was unchanged (Table 1). Under anaerobic conditions, the antimicrobial activity of FOM against strain M68 increased from 12.5 μg/ml to 6.25 μg/ml and the susceptibility of FOM against strain M49 was potentiated as shown by decrease in MIC from 800 μg/ml

Fig. 1. Incorporation of [^{3}H]FOM into *E. coli* cells.

E. coli M 68 (FOM-susceptible) (○, ●) and *E. coli* M 49 (FOM-resistant) (△, ▲). Symbols: absence of (● and ▲) and presence of 5 mM G6P (○ and △).



to 200 µg/ml when compared with the MICs under aerobic condition. The MICs of strain M68 showed the same value in three independent experiments. FOM resistant strains isolated *in vitro* are usually chromosomal mutants impaired in the Uhp and GlpT uptake systems[2,3,23,24]. On the other hand, it has been reported that FOM resistance in mutants is dependent on the cAMP-mediated GlpT system that is regulated by *ptsI*[13], *glp*[12], *crp* (cAMP receptor protein)[12], and *cyaA*[12], or to Uhp system or to *murA* (*murZ*)(UDP-GlcNAc-3-*O*-enolpyruvyl transferase)[25,26]. Accordingly, we suggest that these resistant strains have an impairment in the GlpT system. Our results suggest that other FOM resistance determinants, in addition to the Uhp system, may exist in strain M49 as the MIC remained at 200 µg/ml under anaerobic condition.

[^{3}H]FOM was more actively incorporated into strain M68 than into strain M49 (Fig. 1), and strain M68 showed increased uptake in the presence of 5 mM G6P. Accordingly, it was confirmed that strain M49 has an impaired Uhp system.

In conclusion, the results of the present study indicate that the distribution of FOM MIC of *E. coli* strains in Japan has not changed between 1975 and 1996, and that plasmid-encoded FOM resistant strain in clinical isolates of *E. coli* was not detected in Japan in 1996. Furthermore, it was shown that the mechanism for development of FOM-resistance in one *E. coli* resistant isolate M49 was due to impairment of uptake system(s).

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