International Journal of Medical Microbiology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

journal homepage: www.elsevier.com/locate/ijmm

Biological cost of fosfomycin resistance in *Escherichia coli* in a murine model of urinary tract infection

A. Pourbaix[a,b,1], F. Guérin[c,d,1], V.de Lastours[a,b,e], F. Chau[a,b], M. Auzou[c,d], E. Boulley[a,b], V. Cattoir[c,d], B. Fantin[a,b,e,*]

[a] INSERM, IAME, UMR 1137, F-75018 Paris, France
[b] Université Paris Diderot, IAME, UMR 1137, Sorbonne Paris Cité, F-75018 Paris, France
[c] Université de Caen Normandie, EA 4655 (équipe « Antibio-résistance »), F-14032 Caen, France
[d] CHU de Caen, Service de Microbiologie, F-14033 Caen, France
[e] AP-HP, Groupe Hospitalier Paris Nord Val de Seine, Hôpital Beaujon, Service de médecine interne, F-92210 Clichy, France

ARTICLE INFO

Keywords:
E. coli
UTI
Fosfomycin
Antibiotic resistance
Fitness
Virulence

ABSTRACT

Prevalence of fosfomycin resistance in *E. coli* clinical isolates from UTIs remains very low. Our hypothesis was that fosfomycin resistance may be associated with a biological cost. Three groups of strains of *E. coli* belonging to the B2 phylogenetic group were used: clinical wild-type (WT) isolates, clinical multidrug-resistant isolates and *in vitro* fosfomycin-resistant derivatives from the uropathogen clinical strain *E. coli* CFT073. In each group fosfomycin-susceptible and −resistant isolates were compared. *In vitro*, we found a significantly decreased growth rate for fosfomycin-resistant strains as compared with susceptible strains in the WT group. In a murine model of ascending UTI, there was a significant reduction in infection rates with fosfomycin-resistant isolates as compared with susceptible ones, in all 3 study groups, ranging from 28 to 39% ($P < 0.03$). All fosfomycin-susceptible clinical strains were virulent *in vivo* (13/13), while fosfomycin-resistant clinical strains were either virulent (2/7) or non-virulent (5/7) ($P < 0.002$). This difference was not explained by the number of virulence factors or pathogenicity-associated islands. In conclusion, fosfomycin resistance appears to carry some biological cost in *E. coli*, which may explain in part the apparent paradox of the low prevalence of fosfomycin resistance despite a high rate of spontaneous mutants.

1. Introduction

Over the last decades, resistance to β-lactams among Enterobacteriaceae has emerged as a major public-health threat. Isolates of *Escherichia coli* producing extended-spectrum β-lactamase (ESBL), especially of the CTX-M type, are currently responsible for a large proportion of urinary tract infections (UTIs), in the community as well as in the healthcare setting (Coque et al., 2008; Livermore et al., 2007; Nicolas-Chanoine et al., 2013).

In an era of increasing levels of antibiotic resistance, fosfomycin has attracted renewed interest for the treatment of infections caused by multidrug resistant (MDR) pathogens, and especially UTIs. Indeed, it has a broad spectrum antimicrobial activity and a favorable safety profile with a relatively minor impact on the intestinal microbiota (Falagas et al., 2016). Fosfomycin-tromethamine, a soluble salt with improved bioavailability over fosfomycin, is currently recommended in single-dose as the first-line drug for the treatment of uncomplicated UTIs in Europe (Naber et al., 2001; SPILF, 2015) and in the United State (Gupta et al., 2011). Mechanisms of resistance to fosfomycin consist of modifications in the target protein MurA (leading to reduced affinity to fosfomycin), mutations in the transporter genes (*glpT* and *uhpT*) or their regulatory genes (*uhpA*, *ptsI*, *cyaA*) that allow the active import of the drug into the bacterial cell, or acquisition of fosfomycin-modifying enzymes encoded by plasmid-mediated genes (*fosA*, *fosB*, *fosX*) (Castañeda-García et al., 2013).

The selection of fosfomycin-resistant mutants occurs at very high rates *in vitro* (between 10^{-7} to 10^{-6} cells among Gram-negatives) (Karageorgopoulos et al., 2012), explaining why it is recommended to always use fosfomycin in combination with another antimicrobial agent for the treatment of infections other than uncomplicated UTIs. Additionally, it has been estimated that resistance to fosfomycin during the treatment of an UTI occurs in only ca. 1% of cases (Nilsson et al., 2003). Consequently, the prevalence of fosfomycin resistance should be expected to be high among clinical isolates associated with UTIs, such

* Corresponding author at: Pr Bruno Fantin, Hôpital Beaujon, Service de médecine interne, 100 boulevard du général Leclerc, F-92110 Clichy, France.
E-mail address: bruno.fantin@aphp.fr (B. Fantin).
[1] Both authors equally contributed to this work and may be considered as first authors.

http://dx.doi.org/10.1016/j.ijmm.2017.09.019
Received 12 June 2017; Received in revised form 28 September 2017; Accepted 28 September 2017

Table 1
Primers used in this study.

Primers	Nucleotide sequences (5′ to 3′)	Genes	Length (bp)	Purpose
*murA*_F	ATGGATAAATTTCGTGTTCAGG	*murA*	1260	Sequencing
*murA*_R	TTATTCGCCTTTCACACGCTC	*murA*		Sequencing
*glpT*_F	GCGAGTCGCGAGTTTTCATTG	*glpT*	1359	Sequencing
*glpT*_R	GGCAAATATCCACTGGCACC	*glpT*		Sequencing
*uhpT*_F	TTTTTGAACGCCCAGACACC	*uhpT*	1667	Sequencing
*uhpT*_R	AGTCAGGGGCTATTTGATGG	*uhpT*		Sequencing
*cyaA*1_F	CAGGCGCGAAAAGTGGTAAC	*cyaA*	1338	Sequencing
*cyaA*1_R	GCTAAGGTTATTGCGACGCG	*cyaA*		Sequencing
*cyaA*2_F	GCGTCGTGAAGTGTTAAGCC	*cyaA*	1695	Sequencing
*cyaA*2_R	AGGTATGGCTGGCAACCAAA	*cyaA*		Sequencing
*ptsI*_F	GAAAGCGGTTGAACATCTGG	*ptsI*	1908	Sequencing
*ptsI*_R	TCCTTCTTGTCGTCGGAAAC	*ptsI*		Sequencing
*uhpA*_F	CAGTAGCACCGGCCAGTATC	*uhpA*	729	Sequencing
*uhpA*_R	GCTGCCAGCGTTTTTAATGA	*uhpA*		Sequencing
*fosA3*_F	GCGTCAAGCCTGGCATTT	*fosA3*	282	Identification
*fosA3*_R	GCCGTCAGGGTCGAGAAA	*fosA3*		Identification
*fosA3*_Fc	AATGCGCTTTTTAGCCGGTG	*fosA3*	609	Cloning
*fosA3*_Rc	ACGCTCAGAAGCTCAACGAA	*fosA3*		Cloning

F: Forward, R: Reverse.

as what has occurred with fluoroquinolones. However, despite its massive use for many years in several countries, especially in Europe, the prevalence of fosfomycin resistance in clinical isolates of Enterobacteriaceae responsible for UTIs remains very low, below 2% among *E. coli* isolates in most settings (Rossignol et al., 2017; Karlowsky et al., 2014; Schito et al., 2009). Even among MDR Enterobacteriaceae, levels of susceptibility to fosfomycin remain as high as 80% in ESBL-producing isolates (Martin et al., 2016; Falagas et al., 2010; Östholm Balkhed et al., 2013; Lee et al., 2012; de Cueto et al., 2006). This paradox may be due in part to a significant biological cost associated with fosfomycin resistance, such as decreased *in vitro* growth rate (reduced fitness) and/or attenuated virulence *in vivo* (Nilsson et al., 2003; Marchese et al., 2003). The effect of resistance mutations on fitness is of particular importance for uropathogens because of the limits that the bladder dynamics pose on the growth rate needed to establish an infection (Gordon and Riley, 1992).

Therefore, the aim of this study was to evaluate the impact of the acquisition of fosfomycin resistance mutations on *E. coli in vitro* growth and *in vivo* virulence in an ascending UTI murine model.

2. Materials and methods

2.1. Bacterial strains

Three groups of bacterial strains of *E. coli* were used in this work. The first two groups consisted of clinical isolates from UTIs having occurred in patients between 2012 and 2016 in two French university hospitals. We determined the phylogenetic group of each strain and only used phylogenetic group B2 strains in this work in order to have the most homogenous population of strains, since B2 strains are as most frequently responsible for UTIs in humans and carry the most important virulence factors (Clermont et al., 2000; Tenaillon et al., 2010).

After complete antibiotic susceptibility testing, we determined two groups of strains: the wild-type (WT) group included clinical isolates of *E. coli* that were susceptible to all other antibiotic families and either susceptible or resistant to fosfomycin; the MDR group included *E. coli* clinical isolates that were all ESBL producers and were also resistant to fluoroquinolones, cotrimoxazole and gentamicin, and otherwise susceptible or resistant to fosfomycin.

Finally, an isogenic model was made of a third group of strains which were derivatives of the reference wild-type *E. coli* CFT073 (O6:K2:H1) strain (Mobley et al., 1990). CFT073 was previously used in a murine model of pyelonephritis developed by our group (Labat et al., 2005; Lepeule et al., 2012). *In vitro* fosfomycin-resistant strains (strains C1 to C5) were isolated by plating *E. coli* CFT073 on Mueller-Hinton agar (MH) plates with 32 or 128 µg/mL of fosfomycin (Sanofi-Aventis, Paris, France) and 25 µg/mL of glucose-6-phosphate (Sigma–Aldrich, Saint-Quentin Fallavier, France). We also constructed *E. coli* CFT073 strain with a plasmid which carried the *fosA3* gene (strain C6), using the plasmid pCR-blunt II-TOPO (Life Technologies, Saint-Aubin, France), as previously described, and resulting in CFT073-*fosA3* and its control CFT073-pTOPO harboring the empty vector (Alexandre et al., 2016).

2.2. In vitro fosfomycin activity

MICs of fosfomycin were determined by the agar dilution method in accordance with CLSI and EUCAST guidelines (NCCLS, 2015; EUCAST, 2016), with 25 µg/mL glucose-6-phosphate (G6P) in the medium. The current susceptibility breakpoint of fosfomycin for *Enterobacteriaceae* is a minimum inhibitory concentration (MIC) $\leq$32 µg/mL according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [EUCAST 2016]. Each *in vitro* experiment was performed at least three times.

2.3. Mechanisms of fosfomycin resistance

Mechanisms of fosfomycin resistance were determined for each strain exhibiting an MIC of fosfomycin $\geq$4 µg/mL. Mutations in the genes usually involved in fosfomycin resistance (*murA*, *glpT*, *uhpT*, *cyaA*, *ptsI*, *uhpA*) were determined by nucleotide sequencing after amplification by PCR with the primers presented in Table 1. The amino acid sequences were compared with those of *E. coli* CFT073 and K-12. The *fosA3* gene was also detected by PCR (primers in Table 1).

2.4. Virulence genes

The presence of 24 virulence genes representative of the main classes of *E. coli* extra-intestinal virulence determinants was screened by PCR as previously described (Johnson et al., 2006), including adhesins (*papC*, *papGII*, *papGIII*, *sfa/foc*, *iha*, *hra*, and *ibeA*), toxins (*hlyC*, *cnf1*, and *sat*), iron capture systems (*fyuA*, *irp2*, *iroN*, *iucC*, and *ireA*), protectins (*neuC*, *ompT*, and *traT*), determinant for type 1 pili (*fim1)*, fimbrial adhesins *(afa/dr)*, K1 capsule (*neuB)*, K5 capsule *(kfiC)*, as well as a gene encoding an uropathogenic-specific protein, *usp*. We deduced the presence of 6 pathogenicity-associated islands (PAIs) from the presence of the individual virulence genes (Lefort et al., 2011). For each isolate, we determined the number of virulence factors and the number of PAIs.