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Contribution of hypermutation to fosfomycin heteroresistance in *Escherichia coli*

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Objectives: To explore the effect of combining defects in DNA repair systems with the presence of fosfomycin-resistant mechanisms to explain the mechanisms underlying fosfomycin heteroresistance phenotypes in Enterobacteriaceae.

Materials and methods: We used 11 clinical *Escherichia coli* isolates together with isogenic single-gene deletion mutants in the *E. coli* DNA repair system or associated with fosfomycin resistance, combined with double-gene deletion mutants. Fosfomycin MICs were determined by gradient strip assay (GSA) and broth microdilution (BMD). Mutant frequencies for rifampicin (100 mg/L) and fosfomycin (50 and 200 mg/L) were determined. Using two starting inocula, *in vitro* fosfomycin activity was assessed over 24 h in growth (0.5–512 mg/L) and time–kill assays (64 and 307 mg/L).

Results: Strong and weak mutator clinical isolates and single-gene deletion mutants, except for Δ*uhpT* and Δ*dnaQ*, were susceptible by GSA. By BMD, the percentage of resistant clinical isolates reached 36%. Single-gene deletion mutants showed BMD MICs similar to those for subpopulations by GSA. Strong mutators showed a higher probability of selecting fosfomycin mutants at higher concentrations. By combining the two mechanisms of mutation, MICs and ranges of resistant subpopulations increased, enabling strains to survive at higher fosfomycin concentrations in growth monitoring assays. In time–kill assays, high inocula increased survival by 37.5% at 64 mg/L fosfomycin, compared with low starting inocula.

Conclusions: The origin and variability of the fosfomycin heteroresistance phenotype can be partially explained by high mutation frequencies together with mechanisms of fosfomycin resistance. Subpopulations should be considered until clinical meaning is established.

Introduction

Increasing antibiotic resistance rates in Gram-positive and Gram-negative pathogens mean that it is critical to implement alternative treatment strategies. As a result of the limited availability of novel antimicrobial compounds, one of these strategies is to re-evaluate old antimicrobial agents. Fosfomycin, which is currently recommended as oral treatment for uncomplicated urinary tract infections (UTIs), has attracted interest because of its activity against MDR Enterobacteriaceae.[1]

A recent study by our group showed that fosfomycin resistance occurs in a stepwise manner, depending on the metabolic or signalling pathways affected.[2] Fosfomycin susceptibility testing currently requires the addition of glucose-6-phosphate (G6P) to activate rapid fosfomycin intake via the UhpT transporter.[3,4] This methodology, however, masks other mutations relevant to fosfomycin resistance, such as loss of the glycerol-3-phosphate transporter (GlpT), the other transmembrane fosfomycin transporter, or loss of components of the sugar phosphotransferase system PTS (PtsI).[2] These considerations make fosfomycin susceptibility testing results highly dependent on multiple factors, such as bacterial growth, metabolic conditions and the existence of silent mutations with an impact on fosfomycin resistance.[5]

In addition to this, hypermutable (or mutator) microorganisms have increased spontaneous mutation rates as a result of defects in the DNA repair or error avoidance systems. Strains with highly elevated mutation rates readily evolve in natural and laboratory bacterial populations.[6,7] In clinical settings, various studies of a wide variety of aetiological agents, such as *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, among others, have shown the prevalence of mutators in as many as 1%–60% of patients.[8,9] Molecular characterization of these isolates has discovered that the genetic basis of hypermutability includes alterations in the oxidized guanine (GO), methyl-directed mismatch repair (MMR) and nucleotide excision repair (NER) systems, among others.

Antimicrobial heteroresistance is a phenotypic phenomenon, often with unknown genotypic backgrounds, and its definition therefore is both heterogeneous and controversial. El-Halfawy and Valvano[10] defined it as the presence of a subpopulation of cells with the ability to grow at antibiotic concentrations at least 8-fold higher than the highest concentration that does not affect replication of the dominant population. Nicoloff *et al.*[11] recently demonstrated, for several antimicrobial agents such as β-lactams, trimethoprim/sulfamethoxazole or aminoglycosides, but not fosfomycin, that the high prevalence of antibiotic heteroresistance in pathogenic bacteria is a phenomenon mainly caused by spontaneous tandem amplification, typically involving known resistance genes. The prevalence of fosfomycin heteroresistance in a collection of clinical isolates of carbapenem-resistant Enterobacteriaceae and cephalosporin-resistant *E. coli* was shown to be 41.1% and 5%, respectively.[12,13] Nevertheless, the molecular mechanisms underlying the fosfomycin heteroresistance phenotype and their potential role in therapeutic failure are not known.

The aim of the present study was to delve into the different mechanisms involved in fosfomycin resistance, in particular the contribution of the hypermutation state to the phenotype of fosfomycin heteroresistance.

Materials and methods

Bacterial strains

Twelve *E. coli* (derived from the BW25133 strain) single-gene deletion mutants associated with the DNA repair system in *E. coli* (Δ*dnaQ*, Δ*mutH*, Δ*mutL*, Δ*mutM*, Δ*mutS*, Δ*mutT*, Δ*mutY* and Δ*uvrD*) or with fosfomycin resistance (Δ*glpT*, Δ*uhpT*, Δ*cyaA* and Δ*ptsI*) were selected from the KEIO collection.[14] Thirty-two double-gene deletion mutants were generated by phage P1*vir* transduction [Coli Genetic Stock Center (CGSC), Yale University] as described.[15] All gene deletions (single and double) were confirmed by PCR and sequencing, using the specific primers listed in Table S1 (available as Supplementary data at *JAC* Online).

In addition, a total of 11 *E. coli* isolates (C31, C59, C61, C74, P4, P17, P36, P39, P44, P45 and P56) from human samples (four commensal isolates from faeces and seven pathogens isolated from UTIs) were selected for their range of mutator phenotype.[16] The clinical isolates belonged to different *E. coli* phylogenetic groups (phylogroup A: C31, C59, P4, P17, P39 and P56; phylogroup B1: C61 and C74; phylogroup B2: P44 and P45; and phylogroup D: P36). *E. coli* ATCC 25922, BW25113 and/or MG1655 were used as control strains for the different experiments.

Estimation of rifampicin and fosfomycin mutant frequency

Spontaneous fosfomycin- and rifampicin-resistant mutant frequencies were determined for the 11 clinical isolates and the 12 single-gene deletion mutants associated with the bacterial DNA repair system and fosfomycin resistance. WT *E. coli* BW25113 and MG1655 were included as control strains. To minimize the possibility of mutants in the initial culture, an initial inoculum of $\sim 1\times10^3$ cfu/mL was incubated overnight in Mueller–Hinton II broth (MHB) and subsequently spread onto drug-free plates (total bacterial concentration) and Mueller–Hinton agar II plates (MHA) supplemented with 100 mg/L rifampicin, or MHA-G6P (25 mg/L) plates supplemented with fosfomycin at concentrations of 50 and 200 mg/L (subpopulations able to grow at these antimicrobial concentrations). Plates were incubated at 37°C for 24 h. Experiments were performed in quadruplicate. Laboratory strains and clinical isolates were classified based on rifampicin mutant frequency as weak ($<1\times10^{-7}$) or strong ($\geq1\times10^{-7}$) mutators. Pearson's correlation coefficient was estimated to measure the statistical relationship between the frequencies of mutants resistant to fosfomycin and rifampicin.

Susceptibility tests

Fosfomycin MICs in clinical isolates and laboratory strains were determined using broth microdilution (BMD) and gradient strip assay (GSA). BMD was performed using EUCAST recommendations. For BMD, the presence of skipped wells was recorded and considered as subpopulation regrowth.

GSA was performed in duplicate by streaking a 0.5 McFarland inoculum onto MHA, followed by the application of fosfomycin test strips (bioMérieux), and then incubated at 37°C for 24h. *E. coli* BW25113 and ATCC 25922 were used as controls. The MIC value at the intersection of the strip and the main bacterial population zone of inhibition was recorded. The main bacterial population was considered as the uniform dense biomass observed on the plate by naked eye. MICs for subpopulations or spotted colonies with increased MICs were also recorded. Spotted colonies were considered as the appearance of distinct colonies growing within the clear zone of inhibition in the GSA. EUCAST recommendations and susceptibility breakpoints were followed.[3] Pearson's correlation coefficient was estimated to measure the statistical relationship between the MIC observed by BMD with respect to that observed by GSA including or not the bacterial subpopulations within the inhibition zone.

WGS analysis

WGS analysis of the 11 clinical isolates was performed. Translated nucleotide sequences were compared with WT amino acid sequences from DNA repair system proteins (DnaQ, MutH, MutL, MutM, MutS, MutT, MutY, RecA, RecF, RecO, RecR, UvrA, UvrB, UvrC, UvrD and UvrY) and proteins involved in fosfomycin resistance (Crp, CyaA, GlpT, UhpT, PtsI, UhpA, UhpB, UhpC and UhpT). All these steps are detailed in the Supplementary Materials and methods. Synonymous mutations were not recorded. Absence of the *mutS* gene in some clinical isolates was confirmed by Southern blotting (see Supplementary Materials and methods).

Subpopulation growth monitoring

All clinical isolates and single- and double-mutant strains were monitored for subpopulation growth. The starting inoculum was $\sim 5\times10^5$ cfu/well. Bacterial strains were grown in 96-well flat bottom plates with MHB containing 25 mg/L G6P alone (controls) and a range of fosfomycin concentrations from 0.5 to 512 mg/L in 2-fold dilutions. Bacterial growth over time was monitored by measuring OD at 595 nm every 60 min for 24 h at 37°C using the microplate reader Infinite 200Pro (Tecan Group AG, Männedorf, Switzerland). The limit of detection was 0.08, which is equivalent to a cell density of 1×10^8 cfu/mL. Assays were performed in triplicate. Bacterial viability percentages for each well were determined by comparing OD values at 24 h with the control well (100% viability).

In vitro fosfomycin activity using time–kill curves

In vitro fosfomycin activity was assessed by time–kill curves with two different starting bacterial inocula: a high inoculum with 1×10^7 cfu (5×10^5 cfu/mL in 20 mL) and a low inoculum of 1×10^5 cfu (5×10^3 cfu/mL in 20 mL). To evaluate the role of bacterial subpopulations after fosfomycin exposure at clinically significant concentrations, fosfomycin concentrations of 64 mg/L (lowest concentration in the resistance category according to current EUCAST breakpoints) and 307 mg/L (mean maximum plasma concentration in humans observed at steady-state after a dose of fosfomycin 8 g/q8h)[17] were tested. Experiments were performed in MHB with G6P, and bacterial growth was quantified at 0, 2, 4, 8 and 24 h after incubation, with shaking at 37°C. Samples were washed in saline to avoid the carryover effect, then diluted and plated onto MHA plates (total viable population) and MHA plates supplemented with 25 mg/L G6P and 64 mg/L fosfomycin (viable resistant population). The limit of detection was 1.3 $\log_{10}$ cfu/mL.

When growth was observed after 24 h, up to five colonies were selected to assess the fosfomycin MICs using GSA. The isolates were serially passaged three times on fosfomycin-free plates to assess the stability of the phenotype.

Results

Estimation of rifampicin and fosfomycin mutant frequency

Figure 1(a) shows the results of mutant frequency estimation, and Figure 1(b) the correlation between rifampicin and fosfomycin mutant frequencies.

Overall, strong mutators (strains with mutant frequencies for rifampicin $\geq1\times10^{-7}$) showed a higher probability of selecting fosfomycin mutants at higher concentrations. All clinical isolates with mutant frequencies of $\geq1\times10^{-7}$ for rifampicin showed fosfomycin-resistant mutants able to grow at 50 mg/L fosfomycin, while C59, C61, C74 and P45 grew at 200 mg/L fosfomycin. On the other hand, among the single-gene deletion mutants classified as strong mutators using this breakpoint, neither Δ*mutH*, Δ*mutS* nor Δ*mutY* selected resistant subpopulations at fosfomycin concentrations of 200 mg/L.

Furthermore, with the exception of the Δ*mutM* mutant, neither the clinical isolates nor the single-gene deletion mutants with mutant frequencies of $<1\times10^{-7}$ for rifampicin selected fosfomycin-resistant mutants above 50 mg/L.

This single-gene deletion mutant (Δ*mutM*) showed a weak mutator phenotype, similar to clinical isolates C31, P4 and P56, with mean mutant frequencies for rifampicin of approximately 1×10^{-8} (SD range 3×10^{-8} to 1.67×10^{-8}). Of all strains included in this assay, the strain lacking the *dnaQ* gene had the highest mutant frequencies for rifampicin (mean 1.25×10^{-5}; SD 1.09×10^{-5}). In relation to fosfomycin mutant frequencies, the highest values for fosfomycin at 50 mg/L and 200 mg/L were observed in Δ*uhpT* [2.51×10^{-2} (SD 4.99×10^{-2}); 1.35×10^{-6} (SD 7.90×10^{-7})] together with Δ*dnaQ* [5.11×10^{-3} (SD 9.67×10^{-3}); 2.10×10^{-6} (SD 2.11×10^{-6})].

Pearson's correlation analysis identified a significant association between the frequencies of mutants resistant to fosfomycin and rifampicin: rifampicin 100 mg/L versus fosfomycin 50 mg/L ($r=0.76$; 95% CI 0.48–0.9; $P<0.0001$) and rifampicin 100 mg/L versus fosfomycin 200 mg/L ($r=0.75$; 95% CI 0.48–0.89; $P<0.0001$). The deletions in genes associated with fosfomycin resistance had no impact on the frequency of mutants resistant to rifampicin.

Bacterial susceptibility

Fosfomycin MICs for the isogenic collection and clinical isolates are shown in Table 1. By GSA, all strong mutator clinical isolates were susceptible, and with subpopulations within the ellipse of inhibition. The maximum range between the MIC for the main population and subpopulation MICs was observed in C59, C61, C74, P17 and P45 isolates with ≥7 $\log_2$ dilutions of difference. The least difference in MICs between the main population and subpopulations was detected in C31, P4 isolates and control strains with a ≤ 2 $\log_2$ difference. The highest subpopulation MICs (32 mg/L) were for C61 and C74. By BMD, the percentage of clinical isolates considered resistant rose to 36% (4/11), reaching MIC values of up to 256 mg/L (C61 and C74).

Strains with mutations in fosfomycin-related resistance genes, Δ*glpT*, Δ*cyaA* and Δ*ptsI* showed MICs within the susceptible range by BMD and GSA. Δ*glpT* and Δ*ptsI* strains showed similar susceptibility to WT strains using both methods (Figure 2). Against the Δ*uhpT* strain, the MIC was above the susceptibility breakpoint (>32 mg/L). With respect to GSA, DNA repair system mutants showed main population susceptibility of ≤2 mg/L and subpopulations did not grow beyond 32 mg/L, except for the Δ*dnaQ* strain, whose subpopulations had MICs of up to 512 mg/L. Δ*mutS* and Δ*dnaQ* strains were considered resistant by BMD, but not by GSA (Figure 2). Overall, these isolates showed similar MICs by BMD (± 1 $\log_2$ dilution) to those observed for subpopulations using the GSA, except for Δ*mutS* and Δ*mutM* (± 2 $\log_2$ and ± 3 $\log_2$ dilutions, respectively).

Finally, 81.25% (26/32) of the double-gene deletion mutants tested by BMD grew beyond 32 mg/L, being resistant according to this method (Table 1). By GSA, and considering colonies within the inhibition ellipse zone, 75% (24/32) of double mutants reached MIC values above the susceptibility breakpoint. For double mutants considered susceptible (25%, 8/32), three were resistant by BMD. However, when the colonies inside the inhibition zone were ignored, the percentage of resistant strains fell to 18.75% (6/32) and 20 of the double mutants considered susceptible by this method were resistant by BMD. Pearson's correlation analysis identified a significant association between the MIC performed by BMD versus GSA including the scattered colonies ($r=0.78$; 95% CI 0.65–0.86; $P<0.0001$), but not versus the MIC performed by GSA excluding the more resistant subpopulations ($r=0.09$; 95% CI −0.17 to 0.34; $P=0.49$).

WGS

Analyses of translated nucleotide sequences of genes associated with fosfomycin resistance (Table S2 and Figure 3) showed no mutations in Crp, MurA or UhpA proteins in our collection of clinical isolates, except for the C61 isolate, which presented a single polymorphism in UhpA. No deletions or insertions were detected among the rest of the amino acid sequences associated with fosfomycin resistance, although many different polymorphisms were found and some of them were conserved among the clinical isolates.

With respect to proteins involved with the bacterial DNA repair system, only RecA and RecR showed WT sequences. The absence of *mutS* was observed in four isolates (C59, C61, C74 and P36) and a partial deletion was observed in *E. coli* P45, confirmed by PCR sequencing (data not shown) and Southern blotting (Figure S1).

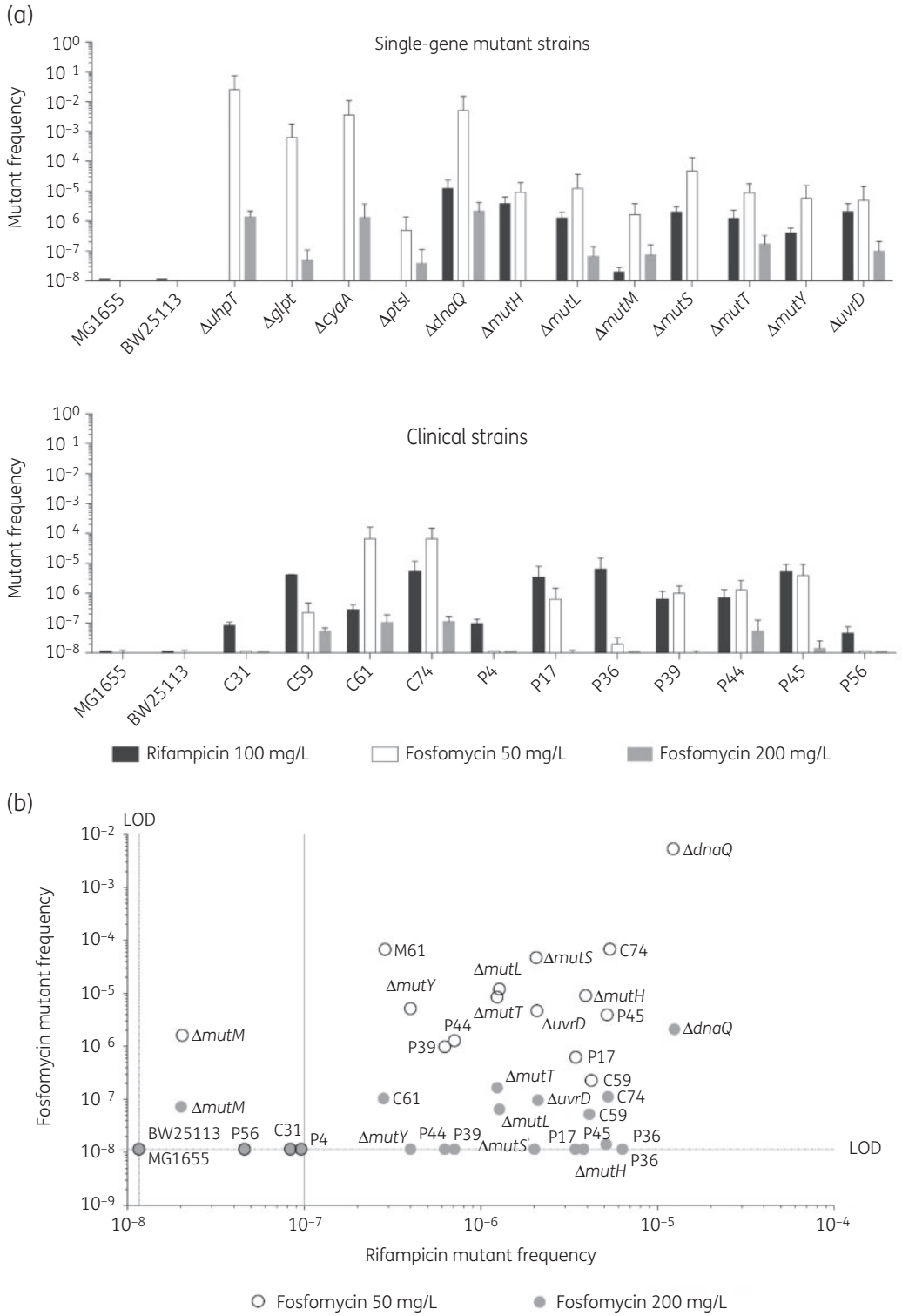

Figure 1. (a) Mutant frequencies for rifampicin at 100 mg/L (black bars) and fosfomycin at 50 mg/L (white bars) and 200 mg/L (grey bars). (b) Correlation between hypermutability (rifampicin mutant frequency at 100 mg/L) and fosfomycin mutant frequency at 50 mg/L (open circles) or 200 mg/L (filled circles); the vertical line separates weak mutators on the left from strong mutators on the right. LOD, limit of detection.

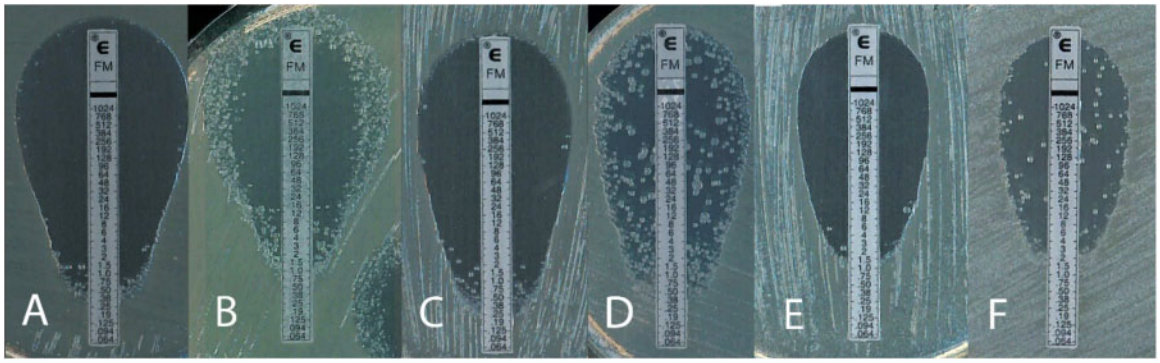

Figure 2. Fosfomycin gradient strip assay for (a) *E. coli* BW25113 WT, (b) Δ*glpT*, (c) Δ*mutS,* (d) Δ*mutS-glpT* strains, (e) Δ*ptsI* and (f) Δ*mutS-ptsI*. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

Table 1. Clinical, single-gene and double-gene mutant MICs (mg/L) tested by BMD and GSA

		GSA	
Strain	BMD	Main population	Subpopulations
ATCC 25922	2	0.50	1.50
BW25113	2	0.50	1.50
C31	2	1	4
C59	16	0.125	16
C61	256	0.25	32
C74	256	0.25	32
P4	1	0.75	2
P17	32	0.06	12
P36	32	0.125	6
P39	8	0.125	12
P44	64	0.25	12
P45	64	0.06	12
P56	4	0.125	1
Δ*dnaQ*	256	1	512
Δ*mutH*	16	2	32
Δ*mutL*	16	1	8
Δ*mutM*	2	1	16
Δ*mutS*	64	1	16
Δ*mutT*	32	1	24
Δ*mutY*	32	1	16
Δ*uvrD*	32	1	16
Δ*glpT*	4 (subp. 128)	0.25	1.5
Δ*uhpT*	128	16	64
Δ*cyaA*	8	12	16
Δ*ptsI*	4	1.5	1.5
Δ*dnaQ-glpT*	≥1024	4	1024
Δ*dnaQ-uhpT*	512	128	1024
Δ*dnaQ-cyaA*	≥1024	3	1024
Δ*dnaQ-ptsI*	≥1024	2	1024
Δ*mutH-glpT*	256	0.5	256
Δ*mutH-uhpT*	256	16	256
Δ*mutH-cyaA*	256	1	128
Δ*mutH-ptsI*	256	1.5	512
Δ*mutL-glpT*	256	1.5	384
Δ*mutL-uhpT*	256	16	256
Δ*mutL-cyaA*	128	2	256
Δ*mutL-ptsI*	256	1.5	256
Δ*mutM-uhpT*	1 (subp. 128)	0.5	128
Δ*mutM-glpT*	128	16	96
Δ*mutM-cyaA*	128	1	4
Δ*mutM-ptsI*	16 (subp. 64)	1	2
Δ*mutS-uhpT*	256	1.5	128
Δ*mutS-glpT*	256	16	512
Δ*mutS-cyaA*	256	12	16
Δ*mutS-ptsI*	512	2	512
Δ*mutT-uhpT*	256	0.5	512
Δ*mutT-glpT*	512	16	256
Δ*mutT-cyaA*	256	12	128
Δ*mutT-ptsI*	512	2	192
Δ*mutY-uhpT*	256	0.75	128
Δ*mutY-glpT*	256	64	1024
Δ*mutY-cyaA*	16	4	8
Δ*mutY-ptsI*	2	0.25	2
Δ*uvrD-uhpT*	256	0.38	384
Δ*uvrD-glpT*	512	12	192
Δ*uvrD-cyaA*	32	1.5	32
Δ*uvrD-ptsI*	32	1	24

subp., subpopulation.

E. coli P44 showed IS*26* in the *mutT* gene (between Ala27 and Arg28).

Clinical isolates with the lowest number of polymorphisms were C31, P4, P17 and P56. *E. coli* P4 was the only isolate with the amino acid sequences equal to the WT strain *E. coli* MG1655. Sequence Read Archive accession numbers (https://www.ncbi.nlm.nih.gov/sra) of the clinical isolates are the following: C31 (SRX7726327), C59 (SRX7726335), C61 (SRX7726333), C74 (SRX7726336), P4 (SRX7726330), P17 (SRX7726334), P36

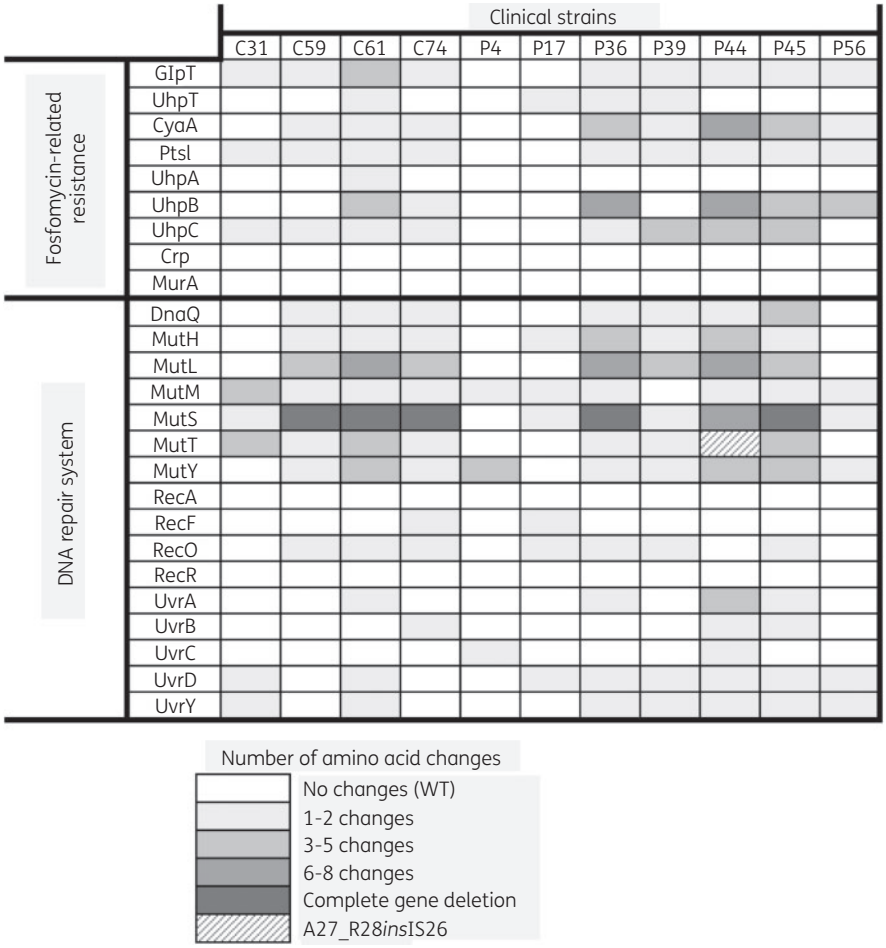

Figure 3. Plot showing the number of mutations observed in proteins related to fosfomycin resistance and DNA repair system in the clinical strains.

(SRX7726329), P39 (SRX7726331), P44 (SRX7726328), P45 SRX7726337) and P56 (SRX7726332).

Bacterial growth monitoring

Figure 4 shows the 24 h growth monitoring assays, expressed as the percentage of viable bacteria at each fosfomycin concentration. The control strains, *E. coli* ATCC 25922 and BW25113, exhibited MICs of 0.5 mg/L and 1 mg/L, respectively. *E. coli* ATCC 25922 showed a subpopulation growing at concentrations of 2 mg/L in one replicate.

Most of the clinical isolates (63.6%, 7/11) were able to grow at fosfomycin concentrations at least eight times higher than control strains, except for C31, P4, P39 and P56 [MICs 2, 1, 8 and 4 mg/L (± 1 $\log_2$), respectively)]. Only C61 and C74 isolates grew beyond 32 mg/L. Among single-gene deletion mutants associated with fosfomycin resistance and DNA repair systems, only Δ*uhpT* and Δ*dnaQ* strains grew above the susceptibility breakpoint. The single-gene deletion mutant Δ*glpT* did not grow beyond 4 mg/L, although one subpopulation grew at a concentration of 128 mg/L.

Finally, most double-gene deletion mutants (87.5%, 28/32) survived at concentrations over the susceptible breakpoint (32 mg/L). Only four of the double mutants (Δ*mutM*-Δ*uhpT*, Δ*mutY*-Δ*glpT*, Δ*mutY*-Δ*cyaA* and Δ*uvrD*-Δ*cyaA*) were not viable above 32 mg/L.

In vitro fosfomycin activity by time–kill curves

The results of the time–kill assays are shown in Figure 5. At the low starting bacterial concentration, none of the strains survived at fosfomycin concentrations of 64 mg/L, except for *E. coli* C74 and the double-mutant laboratory strain, Δ*mutS*-Δ*glpT*. This double mutant was also the only strain able to survive after 24 h in broth culture supplemented with 307 mg/L.

In time–kill assays with high starting inocula, on the other hand, the percentage of strains surviving after 24 h increased from 12.5% (1/8) to 50% (4/8) at fosfomycin concentrations of 64 mg/L compared with those that survived at the low starting inocula. However, even with high initial bacterial concentrations, only C74 and Δ*mutS*-Δ*glpT* strains survived at the maximum fosfomycin concentration tested of 307 mg/L, due to regrowth caused by subpopulations able to grow at the selecting concentration of 64 mg/L fosfomycin.

Fosfomycin MICs for bacterial isolates that were able to survive at fosfomycin concentrations of 64 and 307 mg/L were between 128 and 1024 mg/L by GSA testing.

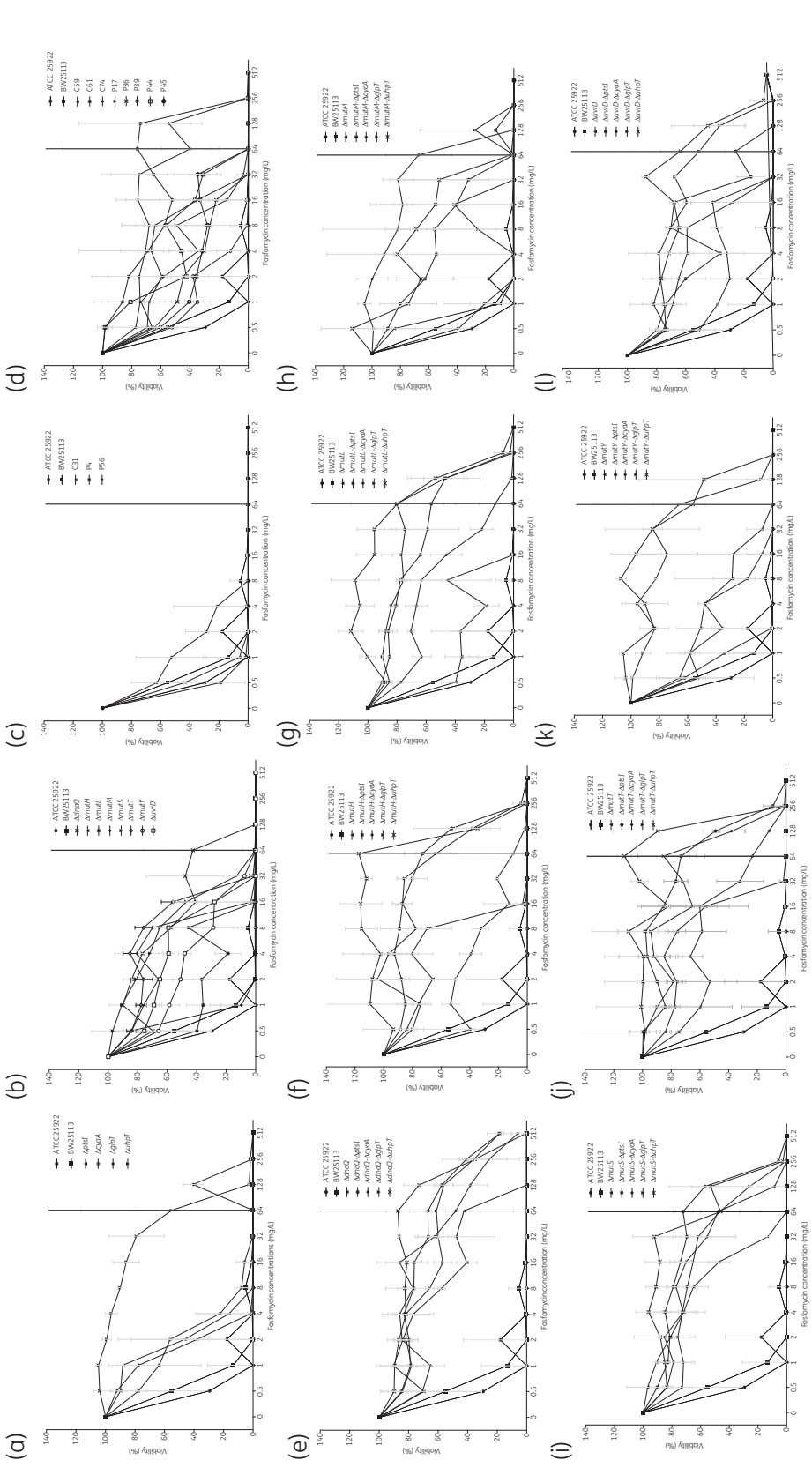

Figure 4. Growth curve analysis in MHB at 24 h with fosfomycin concentrations ranging from 0.5 to 512 mg/L. (a) Fosfomycin-related resistant mutants, (b) DNA repair system mutants, (c) weak mutator clinical isolates, (d) strong mutator clinical isolates and (e–l) fosfomycin-related resistant and DNA repair system double mutants. Vertical lines denote the fosfomycin susceptibility breakpoint.

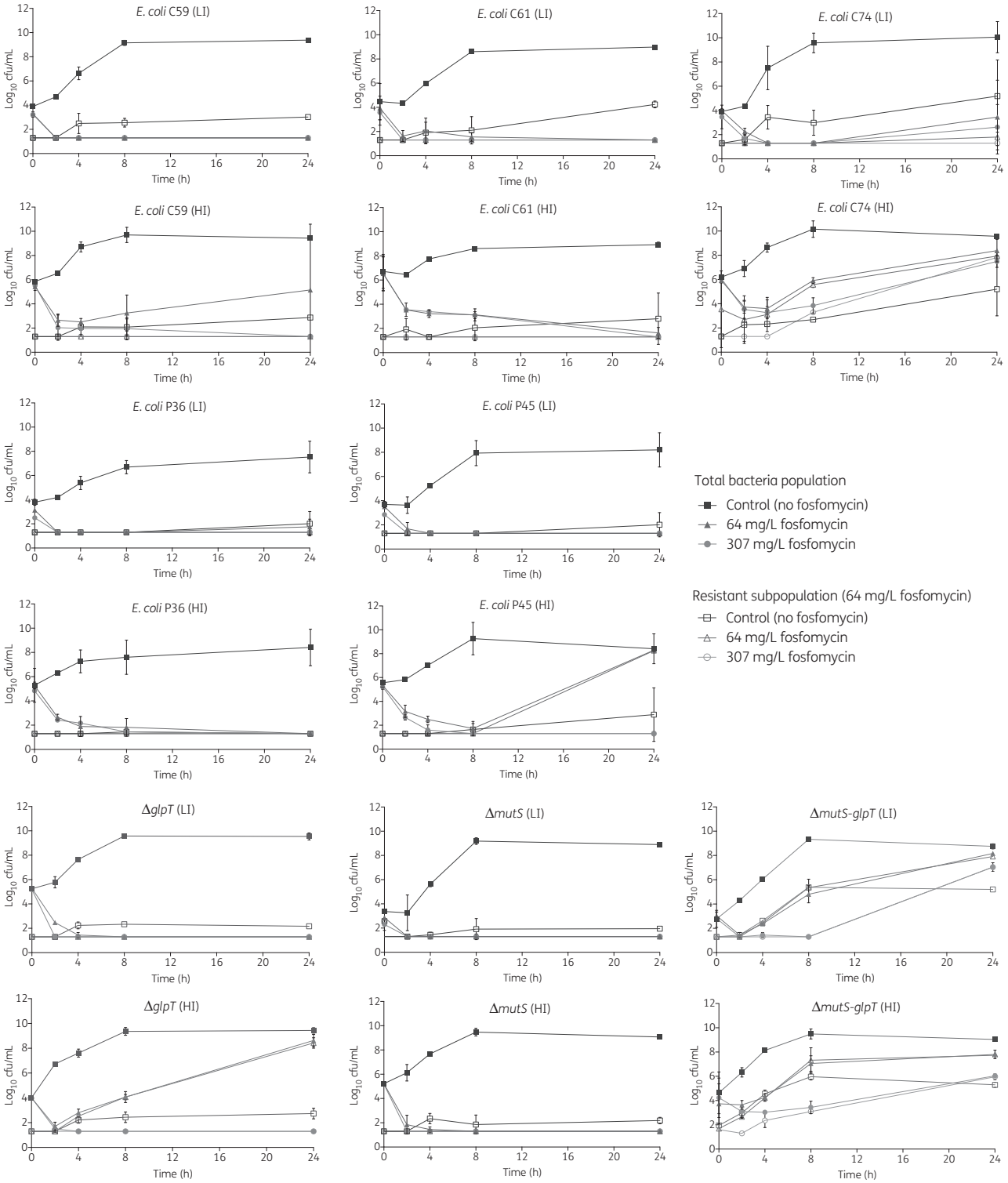

Figure 5. Time–kill curves for *E. coli* clinical isolates, the single-gene deletion mutants Δ*glpT* and Δ*mutS* and the double-gene deletion mutant Δ*glpT-mutS*, using 64 and 307 mg/L fosfomycin and starting bacterial concentrations of 5×10^3 (LI) and 5×10^5 cfu/mL (HI).

Discussion

Using both an isogenic collection of laboratory mutants and clinical isolates, the present study showed that the heterogeneous fosfomycin resistance phenotype can be partly explained by a high mutation rate together with the presence of mutations in genes associated with fosfomycin resistance. Fosfomycin heteroresistance is a common phenotype and can be observed by the presence of colonies inside the inhibition zone in disc diffusion or GSA.[12,13] Nevertheless, the density and spread across the range of antimicrobial concentrations is highly strain dependent and the underlying mechanisms are not well known.

In *E. coli*, and probably other Enterobacteriaceae, fosfomycin resistance increases in a stepwise manner via the acquisition of mutations in genes associated with fosfomycin resistance (such as *glpT*, *uhpT*, *cyaA*, *crp* and *ptsI*).[2] In 'normomutator' strains, i.e. those with a mutation rate of $<4\times10^{-7}$,[18] mutations produce increased but stable MIC values. However, WT strains for resistance genes associated with fosfomycin resistance but with a weak or strong mutator phenotype (i.e. the absence of or defective DNA repair systems) show an increased number of resistant subpopulations, with growth limited to fosfomycin concentrations of 64 mg/L using standard reference methods, which includes the addition of G6P, 25 mg/L for broth dilution or 50 μg for disc diffusion assays.[3] The reason for this is that, even under a strong mutator phenotype, just a single mutational event affects fosfomycin-related resistance genes and, as we have previously shown, the highest MIC observed is produced with the loss of any of the components of the G6P transporter (*uhpT* gene or the two-component system encoded by the *uhpABC* operon).[2] This is true of all mutants involved in mechanisms of the methyl-directed mismatch repair pathway (*mutHLS*), 8-oxyguanine suppression (*mutT*, *mutM* and *mutY*) or nucleotide excision repair (*uvrD*) systems. The only exception was the mutant of the *dnaQ* gene (the epsilon subunit of DNA polymerase III involved in the 3′ to 5′ exonuclease proofreading activity of the holoenzyme), which because of its high mutation rate was able to accumulate mutations in more than one fosfomycin-related resistance gene, so enabling growth at fosfomycin concentrations beyond 64 mg/L.

Hence, under a mutator background, the presence of certain mutations in fosfomycin-related resistance genes, especially those with low impact on the MIC (i.e. *glpT* or *ptsI*), is responsible for the different fosfomycin heteroresistance phenotypes, and subpopulation densities are related to the mutability status of the bacteria (i.e. the ability to acquire more mutations due to the absence of or a defective DNA repair system, for example). This phenomenon was in part shown by Ellington *et al.*[19] where mutator phenotypes were found to have an increased propensity to fosfomycin resistance. Nicoloff *et al.*[11] recently showed that the origin of heteroresistance to multiple antimicrobials, excluding fosfomycin, was based on a transient or stable increase of certain resistance determinants (duplications, etc.) in bacterial subpopulations that are then selected by the presence of relevant antimicrobial concentrations. While some fosfomycin-related genes may cause hypersusceptibility, such as transporter overexpression, we cannot rule out the possibility that transient or stable amplification of other genes could be another source of the heterogeneous response to fosfomycin.

Inaccuracies or low agreement between different methods for testing fosfomycin susceptibility have previously been associated with different starting bacterial concentrations used in assays, which means variation in the chance selection of resistant subpopulations through inoculation of mutants at the start of susceptibility testing.[20,21] In broth-based methods, the presence of bacterial subpopulations frequently does not make it possible to differentiate between uniformly resistant and heteroresistant bacteria. In this respect, agar-based methods such as disc diffusion or GSA are likely to be more effective for the screening of bacterial subpopulations, since minority subpopulations do not replace the susceptible population. We performed fosfomycin susceptibility testing by either BMD or GSA to enhance the importance of including the subpopulations in the interpretation MIC, knowing neither is a reference method but they are commonly used in daily routine in clinical microbiology susceptibility testing.

Since 2017, the EUCAST guidelines have recommended ignoring all spotted colonies inside the inhibition zone in disc diffusion assays, and reading the outer zone edge.[3] This recommendation should be interpreted with some caution because these subpopulations show stable resistance and could lead to therapeutic failures, especially in cases with high bacterial concentrations where subpopulations may be over-represented.

A limitation of the present study is that it was focused on the relationship between high mutation rate and the increase in resistant subpopulations. Nevertheless, we cannot rule out other sources of heteroresistance based on mechanisms different from increased mutation frequency, as previously stated.

In our collection of laboratory mutants, the genotype of our strains explained reasonably well both the fosfomycin heteroresistance phenotype and its expected variability. However, in the clinical isolates that were fosfomycin heteroresistant, when deleterious mutations in fosfomycin-related resistance genes or DNA repair systems were studied by WGS, this correlation was not always found. Since different polymorphisms with unknown roles were found in some isolates, the possibility of other factors affecting fosfomycin heteroresistance cannot be ruled out.

Finally, in the time–kill assays, we used relevant (susceptibility breakpoint) and physiological (plasma C_{max}) fosfomycin concentrations and observed fosfomycin activity with low starting inocula in all strains tested, except for the double-gene mutant Δ*mutS*-Δ*glpT* and the clinical isolate *E. coli* C74. However, fosfomycin activity may be somewhat overestimated because these static concentrations may not reflect the variations observed in human pharmacokinetics, and could regrow, even with low inoculum strains.[22]

As the main conclusion, the origin and variability observed in the phenotype of heteroresistance to fosfomycin can be partly explained by increased mutability in bacterial strains. These subpopulations should therefore be taken into consideration, not only for fosfomycin susceptibility testing, but also for other antimicrobials.

The fosfomycin GSA is a reliable method for observing the variability and strength of heterogeneous resistance to fosfomycin. Fosfomycin MICs performed using the BMD may be more representative of the susceptibility of the whole bacterial population due to the growth of resistant subpopulations, but very susceptible to low inoculum artefacts or the presence of skipped wells, thus leading to incorrect susceptibility interpretations.

The clinical significance of these results for the treatment of infections caused by heteroresistant subpopulations should be evaluated in further studies, considering fosfomycin

pharmacokinetics and pharmacodynamics in hollow-fibre infection or animal models, or even in clinical observational studies.

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Supplementary data

Supplementary Materials and methods, Tables S1 and S2 and Figure S1 are available as Supplementary data at *JAC* Online.

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