Contribution of hypermutation to fosfomycin heteroresistance in *Escherichia coli*

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Running title: Hypermutation and fosfomycin heteroresistance

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SYNOPSIS

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 phenotype in Enterobacteriaceae.

Materials and methods:

We used eleven 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 (GSA) and broth microdilution assay (BMD). Mutant frequencies for rifampin (100 mg/L) and fosfomycin (50 and 200 mg/L) were determined. Using two starting inocula, *in vitro* fosfomycin activity was assessed over 24h 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 higher probability of selecting fosfomycin mutants at higher concentrations. By combining the two mechanisms of mutation, MICs and range 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 to low starting inocula.

Conclusions.

Origin and variability of 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 reevaluate 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 multidrug-resistant Enterobacteriaceae.[1]

A recent study by our group showed that fosfomycin resistance occurs in a stepwise manner, depending on the metabolic or signaling 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 etiologic 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 defined it as the presence of a subpopulation of cells with the ability to grow at antibiotic concentrations at least eight-fold higher than the highest concentration that does not affect replication of the dominant population.[10] Nicolof *et al.* recently demonstrated, for several antimicrobial agents such as beta-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.[11] 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 hypermutation state to the phenotype of fosfomycin heteroresistance.

MATERIAL 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 specific primers listed in table S1.

In addition, a total of 11 *E. coli* isolates (C31, C59, C61, C74, P4, P17, P36, P39, P44, P45, P56) from human samples (four commensal isolates from feces 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, P56; phylogroup B1: C61, C74; phylogroup B2: P44, P45 and phylogroup D: P36). *E. coli* ATCC 25922, BW25113 and/or MG1655 were used as control strains for the different experiments.

Rifampin and fosfomycin mutant frequency estimation

Spontaneous fosfomycin- and rifampin-resistant mutant frequencies were determined for the 11 clinical isolates and the 12 single-gene deletion mutants associated with bacterial DNA repair system and fosfomycin resistance. Wild-type *E. coli* BW25113 and MG1655 were included as control strains. To minimize the possibility of mutants in the initial culture, an initial inoculum of ~10^3 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 rifampin, 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 37ºC for 24 hours. Experiments were performed in quadruplicate. Laboratory strains and clinical isolates were classified based on rifampin mutant

frequency as weak ($<10^{-7}$) or strong ($\geq 10^{-7}$) mutators. Pearson's correlation coefficient was estimated to measure the statistical relationship between the frequencies of mutants resistant to fosfomycin and rifampin.

Susceptibility tests

Fosfomycin MICs in clinical isolates and laboratory strains were determined using BMD and GSA. BMD were performed using EUCAST recommendations. For BMD, the presence of skipped wells was recorded and considered as subpopulations 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 24 hours. *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 of 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 respect to that observed by GSA including or not the bacterial subpopulations within the inhibition zone.

Whole genome sequencing analysis

Whole genome sequence analysis of the eleven clinical isolates was performed. Translated nucleotide sequences were compared with wild-type amino acid sequences

from DNA repair system proteins (DnaQ, MutH, MutL, MutM, MutS, MutT, MutY, RecA, RecF, RecO, RecR, UvrA, UvrB, UvrC, UvrD, 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 material. Synonymous mutations were not recorded. Absence of *mutS* gene in some clinical isolates were confirmed by Southern blotting (see supplementary material).

Subpopulation growth monitoring

All clinical isolates and single- and double-mutant strains were monitored for subpopulation growth. The starting inoculum was ~$5x10^5$ cfu/well. Bacterial strains were grown in 96-well flat bottom plates with MHB containing 25 mg/L of G6P drug-free (controls) and a range of fosfomycin concentrations from 0.5 to 512 mg/L in two-fold dilutions. Bacterial growth over time was monitored by measuring OD at 595 nm every 60 min for 24 h at 37ºC 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 $1x10^8$ cfu/mL. Assays were performed in triplicate. Bacterial viability percentages for each well were determined comparing OD values at 24 h to 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 10^7 cfu ($5x10^5$ cfu/mL in 20 mL) and a low inoculum of 10^5 cfu ($5x10^3$ cfu/mL in 20 mL). To evaluate the role of bacterial subpopulations after fosfomycin exposure at clinically, 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 8g/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 37ºC. 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 of 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

Rifampin and fosfomycin mutant frequency estimation

Figure 1A shows the results of mutant frequency estimation, and Figure 1B the correlation between rifampin and fosfomycin mutant frequencies.

Overall, strong mutators (strains with mutant frequencies for rifampin $\geq 10^{-7}$) showed a higher probability of selecting fosfomycin mutants at higher concentrations. All clinical isolates with mutant frequencies of $\geq 10^{-7}$ for rifampin 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 $<10^{-7}$ for rifampin 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 rifampin of approximately 10^{-8} (standard deviation (SD) range: $3x10^{-8}$-$1.67x10^{-8}$). Of all strains included in this assay, the strain lacking the *dnaQ* gene had the highest mutant frequencies for rifampin (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 correlation analysis identified a significant association between the frequencies of mutants resistant to fosfomycin and rifampin: 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 rifampin.

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 of the main population and subpopulation MICs was observed in C59, C61, C74, P17 and P45 isolates with $\geq 7 \log_2$ 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 $\leq 2\log_2$ 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 wild-type strains using both methods (Figure 2). Against Δ*uhpT* strain, 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 (+/- $1\log_2$ dilutions) 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 twenty of the double-mutants considered susceptible by this method were resistant by BMD. Pearson 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:-0.17-0.34; p=0.49).

Whole genome sequencing

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 C61 isolate, which presented a single polymorphism in UhpA. No deletions or insertions were detected among the rest of aminoacidic 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 wild-type sequences. The absence of *mutS* was observed in four isolates (C59, C61, C74 and P36) and a partial deletion in *E. coli* P45, confirmed by PCR sequencing (data not shown) and Southern blotting (Figure S1). *E. coli* P44 showed insertion sequence *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 aminoacidic sequences equal to the wild-type 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 (SRX7726329), P39 (SRX7726331), P44 (SRX7726328), P45 SRX7726337) and P56 (SRX7726332).

Bacterial growth monitoring

Figure 4 shows the 24h growth monitoring assays, expressed as percentage of viable bacteria at each fosfomycin concentration. The control strains, *E. coli* ATCC 25922 and

BW 25113, 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 hours 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 hours 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 MIC of 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.

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 disk 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*, *ptsI*, etc.).[2] In normomutator strains, that is, those with a mutation rate of $<4x10^{-7}$,[18] mutations produce increased but stable MIC values. However, wild-type strains for resistance genes associated with fosfomycin resistance but with a weak- or strong-mutator phenotype (that is, 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 disk 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

previously showed, 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 (that is, the ability to acquire more mutations due to the absence of or defective DNA repair system, for example). This phenomenon was in part showed by Ellington *et al.* where mutator phenotypes were found to have an increased propensity to fosfomycin resistance.[19] Nicoloff *et al.* recently showed that the origin of heteroresistance to multiple antimicrobials, excluding fosfomycin, was based on transient or stable increase of certain resistance determinants (duplications, etc.) in bacterial subpopulations that are then selected by the presence of relevant antimicrobial concentrations.[11] While some fosfomycin-related genes may cause hypersusceptibility, like 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 has 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 disk 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 test by either BMD or GSA to enhance the importance of including the subpopulations in the interpretation MIC, knowing neither are reference methods but commonly used in daily routine in clinical microbiology susceptibility testing.

Since 2017, the EUCAST guidelines has recommended ignoring all spotted colonies inside the inhibition zone in disk diffusion assay, 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 overrepresented.

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 whole genome sequencing, 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 Cmax) 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, so leading to wrong 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-fiber infection or animal models, or even in clinical observational studies.

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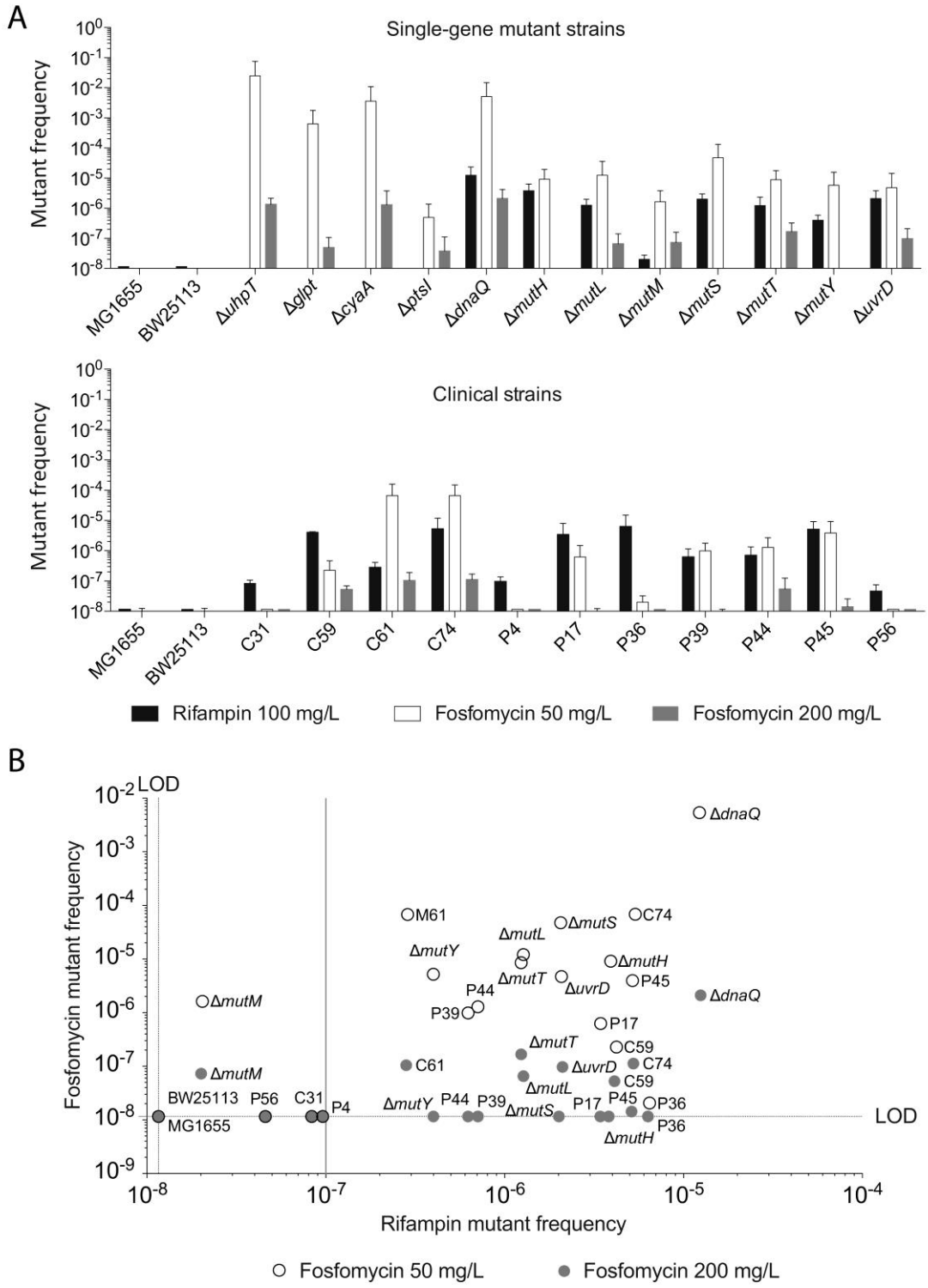
Figure 1. A) Mutant frequencies for rifampin at 100 mg/L (black bars), fosfomycin at 50 mg/L (white bars) and 200 mg/L (grey bars). B) Correlation between hypermutability (rifampin mutant frequency at 100 mg/L) and fosfomycin mutant frequency at 50 mg/L (empty circles) or 200 mg/L (filled circles), vertical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



485 Table 1. Clinical, single-gene and double-gene mutants MICs (mg/L) tested by broth

486 microdilution method (BMD) and gradient strip assay (GSA).

Strains	BMD	GSA		Strains	BMD	GSA	
		Main population	Subpopulations			Main population	Subpopulations
ATCC25922	2	0.50	1.50	Δ***dnaQ-glpT***	≥1024	4	1024
BW25113	2	0.50	1.50	Δ***dna Q-uhpT***	512	128	1024
C31	2	1	4	Δ***dnaQ-cyaA***	≥1024	3	1024
C59	16	0.125	16	Δ***dnaQ-ptsI***	≥1024	2	1024
C61	256	0.25	32	Δ***mutH-glpT***	256	0.5	256
C74	256	0.25	32	Δ***mutH-uhpT***	256	16	256
P4	1	0.75	2	Δ***mutH-cyaA***	256	1	128
P17	32	0.06	12	Δ***mutH-ptsI***	256	1.5	512
P36	32	0.125	6	Δ***mutL-glpT***	256	1.5	384
P39	8	0.125	12	Δ***mutL-uhpT***	256	16	256
P44	64	0.25	12	Δ***mutL-cyaA***	128	2	256
P45	64	0.06	12	Δ***mutL-ptsI***	256	1.5	256
P56	4	0.125	1	Δ***mutM-uhpT***	1 (subp. 128)	0.5	128
Δ***dnaQ***	256	1	512	Δ***mutM-glpT***	128	16	96
Δ***mutH***	16	2	32	Δ***mutM-cyaA***	128	1	4
Δ***mutL***	16	1	8	Δ***mutM-ptsI***	16 (subp. 64)	1	2
Δ***mutM***	2	1	16	Δ***mutS-uhpT***	256	1.5	128
Δ***mutS***	64	1	16	Δ***mutS-glpT***	256	16	512
Δ***mutT***	32	1	24	Δ***mutS-cyaA***	256	12	16
Δ***mutY***	32	1	16	Δ***mutS-ptsI***	512	2	512
Δ***uvrD***	32	1	16	Δ***mutT-uhpT***	256	0.5	512
Δ***glpT***	4 (subp. 128)	0.25	1.5	Δ***mutT-glpT***	512	16	256
Δ***uhpT***	128	16	64	Δ***mutT-cyaA***	256	12	128
Δ***cyaA***	8	12	16	Δ***mutT-ptsI***	512	2	192
Δ***ptsI***	4	1.5	1.5	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

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Figure 2. Fosfomycin gradient strip assay for: A) *E. coli* BW25113 wild-type, 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 printed version of *JAC*.

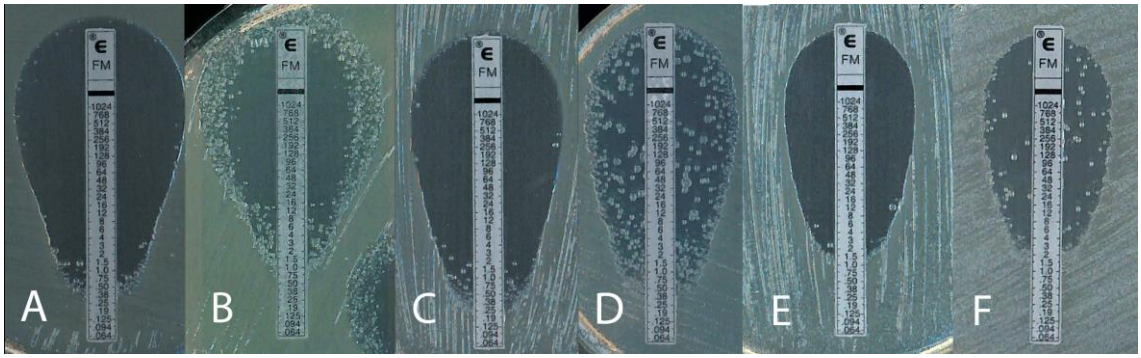


Figure 3. Plot showing the number of mutations observed in proteins related to fosfomycin resistance and DNA repair system in the clinical strains.

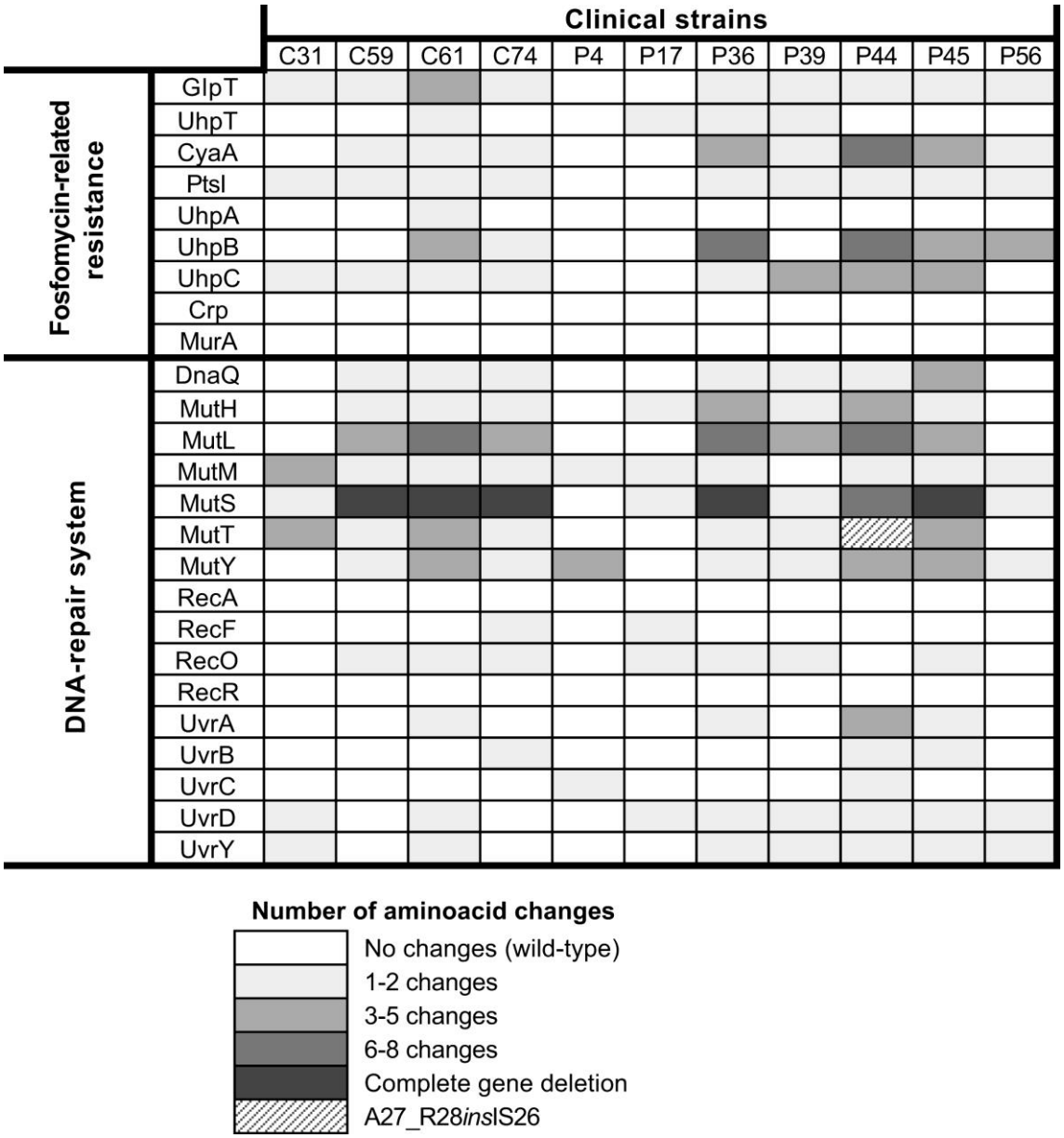


Figure 4. Growth curve analysis in MHB at 24h with fosfomycin concentrations ranging from 0.5 to 512 mg/L. A) Fosfomycin-related resistant mutants, B) DNA repair system mutants, C) Weak mutator clinical isolates, D) Strong mutator clinical isolates, E-L) Fosfomycin-related resistant and DNA repair system double mutants. Vertical lines denote fosfomycin susceptibility breakpoint.

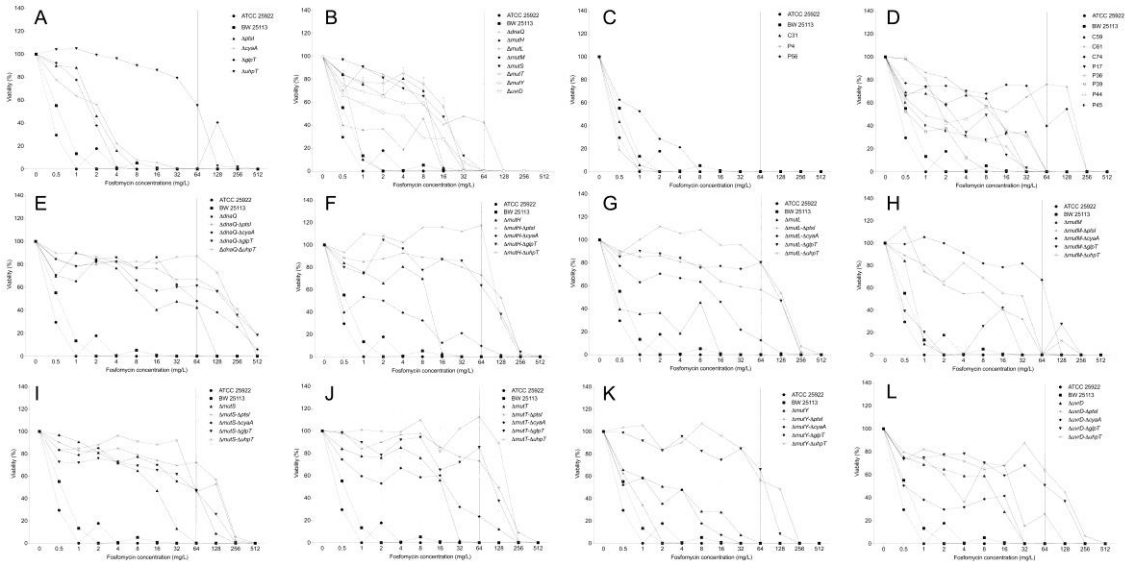
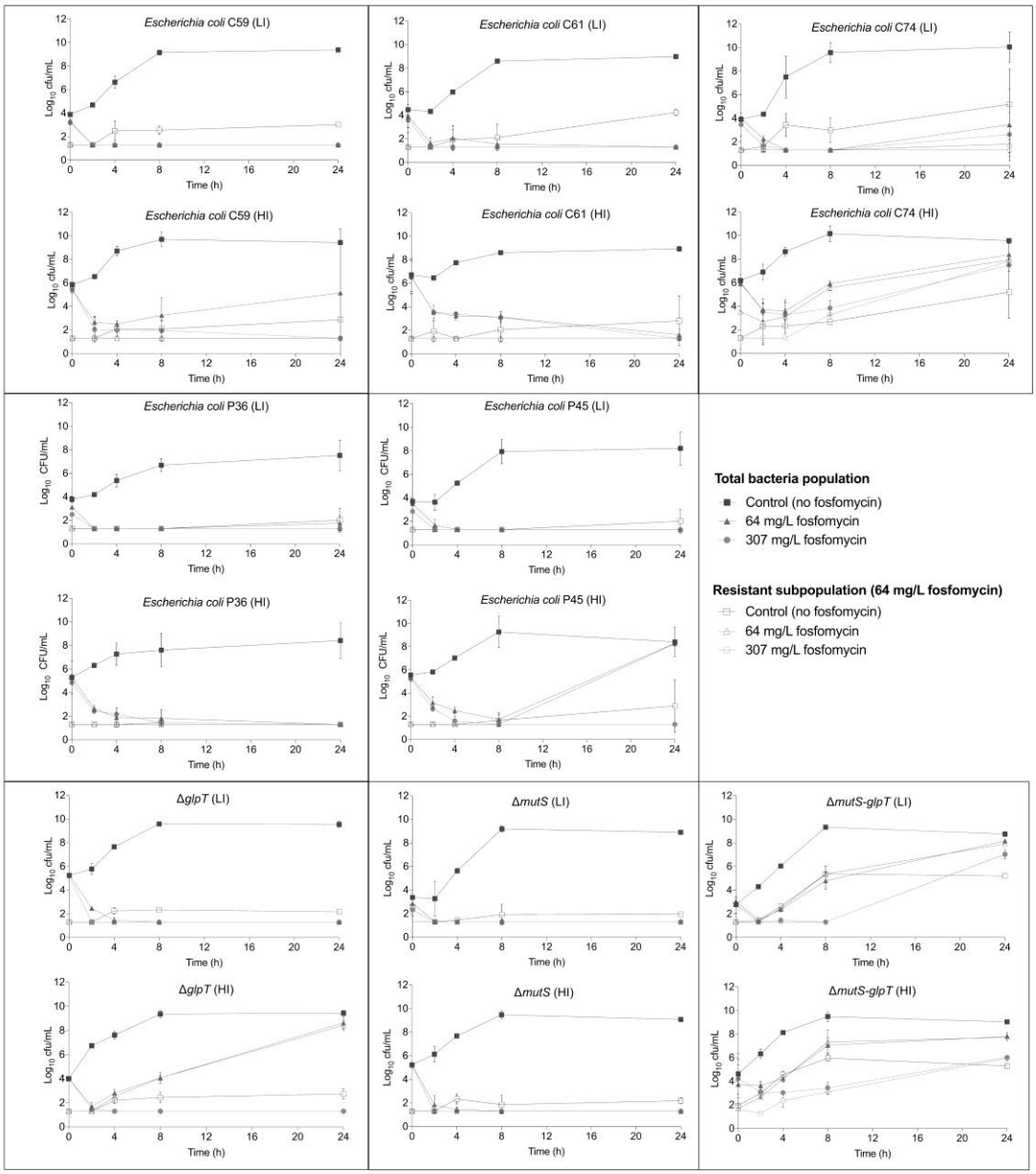


Figure 5. Time–kill curves for *E. coli* clinical isolates and the single-gene deletion mutants Δ*glpT*, Δ*mutS* and the double-gene deletion mutant (Δ*glpT-mutS*), using 64 and 307 mg/L fosfomycin and starting bacterial concentrations of $5x10^3$ (LI) and $5x10^5$ cfu/mL (HI).



SUPPLEMENTARY

MATERIAL AND METHODS.

Table S1. PCR and sequencing primers for mutant confirmation.

Amplified gene	Primer	Sequence	Amplicon size (pb/aa)
glpT	ECglpTF ECglpTR ECglpTintF ECglpTintR	5´-GCGAGTCGCGAGTTTTCATTG-3´ 5´-GGCAAATATCCACTGGCACC-3´ 5´-GGGATGGCCTGGTTCAATGAC-3´ 5´-GCAGTTTGTTCGGCAGTACG-3´	1359/452
uhpT	ECuhpTF ECuhpTR	5´-TTTTTGAACGCCCAGACACC-3´ 5´-AGTCAGGGGCTATTTGATGG-3´	1392/463
cyaA	ECcyaAF ECcyaAR	5´-AACCAGGCGCGAAAAGTGG-3´ 5´-ACCTTCTGGGATTTGCTGG-3´	2547/848
ptsI	ECptsIF ECptsIR	5´-GAAAGCGGTTGAACATCTGG-3´ 5´-TCCTTCTTGTCGTCGGAAAC-3´	1728/575
dnaQ	ECdnaQF ECdnaQR	5´-TTGCCCAGACACGAACCATC-3´ 5´-TTGCCTCGACCTTCGTCAAC-3´	732/243
mutH	ECmutHF ECmutHR	5´-CAGAGAATTGAACAACGCATGTGG-3´ 5´-GCGTTAGGATCGGTTATCCATG-3´	690/229
mutL	ECmutLF ECmutLintF ECmutLR	5´-ACGGCACAAACTGCCAGTAC-3´ 5´-ACACGGCGATCTCACGCTAC-3´ 5´-CGTTAGCCATTGAGCTGCGT-3´	1848/615
mutM	ECmutMF ECmutMR	5´-ATCCAGTTGTTCGCCAGCAC-3´ 5´-GGCGCTGATGGCGAAGTTAG-3´	810/269
mutS	ECmutSF ECmutSintF ECmutSR ECmutSintR	5´-GCGCCTTATGTGATTACAACG-3´ 5´-GGACTTTGTGCTGCCGGTTG-3´ 5´-GGCGATAGTGATGGGCATTG-3´ 5´-TGATTGCGCTGATGGCCTAC-3´	2562/853
mutT	ECmutTF ECmutTR	5´-GAGAGCGCAAAGTAGGACGTAAC-3´ 5´-CAGGCATCGTGTGCGAATGTC-3´	390/129
mutY	ECmutYF ECmutYR	5´-GAAAGTTCCGGTTTACACCCTGC-3´ 5´-TGCAACGTGAAGCAGAAGGTC-3´	1053/350
uvrD	ECuvrDF ECuvrDintF ECuvrDR ECuvrDintR	5´-TGGCATCTCTGACCTCGCTG-3´ 5´-CCAGGATACCAACAACATTCAG-3´ 5´-ATCCGGCCTACATGACGTTG-3´ 5´-CGTACCATGTATGAGCAGGAG-3´	2163/720

Whole genome sequencing analysis

Whole genome sequence analysis of the eleven clinical isolates (C31, C59, C61, C74, P4, P17, P36, P39, P44, P45 and P56) was performed, as follows. Genomic DNA from isolates was extracted from 3 mL bacterial cultures grown to mid-exponential phase in MHB using the DNeasy Blood & Tissue Kit (Qiagen, Netherlands), according to the manufacturer's recommendation. DNA was resuspended in 10 mM Tris-HCl, pH 8.0, and concentrations determined using the Qubit 2.0 fluorometer (Invitrogen, Thermo Fisher Scientific, Massachusetts, USA). In-house Miseq sequencing (Illumina, San Diego, CA, USA) was performed. Libraries were prepared with the Nextera XT DNA library preparation kit (Illumina) and sequencing with a reagent cartridge, V3 600 cycles (Illumina), achieving at least 30x average coverage. CLC Genomic Workbench software (Qiagen, Netherlands) was used for *de novo* assembly of Illumina reads. Bacterial genomes were annotated using the RAST 2.0 annotation server [18]. A database containing wild-type amino acid sequences from DNA repair system proteins (DnaQ, MutH, MutL, MutM, MutS, MutT, MutY, RecA, RecF, RecO, RecR, UvrA, UvrB, UvrC, UvrD, UvrY) and proteins involved in fosfomycin resistance (Crp, CyaA, GlpT, UhpT, PtsI, UhpA, UhpB, UhpC and UhpT) were constructed with sequences obtained from the EcoCyc database [19]. Translated nucleotide sequences from the RAST were compared with our custom database, using the Bioedit Sequence Alignment Editor BioEdit version 5.0.7 (Tom Hall, Ibis Therapeutics, Carlsbad, CA). Protein sequences with <100% identity were analysed using Protein BLAST and mutations were recorded.

All those genes that were not found using this method were subsequently amplified with conventional PCR and sequencing, using specific primers (table S1) to confirm gene deletions. Synonymous mutations were not recorded.

Southern blotting

To confirm the absence of *mutS* gene in clinical isolates, Southern blotting experiments were performed using a digoxigenin-11-dUTP-labeled *mutS* probe. The probe (702 bp) was prepared by PCR amplification (PCR primers: mutSintF: 5′-GGACTTTGTGCTGCCGGTTG-3′; mutSintR: 5′-GCCAACTTTCAGCGTGTCCAG-3′), using the above protocol and replacing the dNTP solution with PCR DIG Labelling Mix (Roche Applied Science, Mannheim, Germany). PCR conditions were adjusted by choosing an annealing temperature of 54 ºC and an extension time of 40s. After PCR, the probe was purified by gel extraction using the QIAquick Gel Extraction Kit (Qiagen). DNA from *E. coli* BW25113 (wild-type) and Δ*mutS* strains were used as positive and negative controls, respectively.

DNA from the control strain and clinical isolates was separated according to size and blotted onto a PVDF membrane filter. The filter was hybridized with the digoxigenin-labelled *mutS* probe.

46 **RESULTS**

47 Table S2. List of non-synonymous mutations found in clinical strains (wt denotes for

48 wild-type)

Genes	C31	C59	C61	C74	P4	P17	P36	P39	P44	P45	P56
GlpT	E448K	L297F E448K	E443Q Q444E E448K	E448K	wt	wt	E448K	L297F E448K	A402V E448K	E448K	E448K
UhpT	wt	wt	A355L E350Q	wt	wt	F160S	E350Q	E350Q	wt	wt	wt
CyaA	wt	N142S	N142S	N142	wt	wt	N142S D837E T840A	N142S	N142S A349E S356K G359E E362D I514V D837E T840A	N142S D837E T840A	N142S
PtsI	R367K	R367K	R367K	E282K R367K	wt	wt	R367K	R367K	A306T R367K	R367K	R367K
UhpA	wt	wt	P25S	wt	wt	wt	wt	wt	wt	wt	wt
UhpB	wt	wt	P84S L85I T166I H482T	S404A	wt	wt	T75M P84S Q441H L456V G459S Q463H H482T	wt	T75M P84S Q441H G459D Q463H H482T	P84S Q441H G459D Q463H H482T	P84S Q441H G495S Q463H H482T
UhpC	Y18H	I108M	Y18H	T435A	*wt*	w*t*	Y18H A177S S417A	Y18H A177S I422A T435A	Y18H A177S S417A	Y18H A177S S417A	wt
Crp	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
MurA	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
DnaQ	wt	I222L Q233E	A200T	I222L Q233E	wt	wt	A200T I222L	I222L	A200T I222L	A101T A200T I222L	wt
MutH	wt	V36A	V36A	V36A	wt	S145R	V36A Q135K V170I	V36A	V36A K79Q Q135K V170I	V36A	wt
MutL	wt	L418P A470V A494G	N131D S350A A368F L418P S459A A494G	L418P P423T A494G	wt	wt	D129N N131D S350A A368V T377S L418P A477V A494G	A73_R74*ins*LA L418P	N131D S350A A368V T377S P389S L418P A477V A494G	N131D P203S G281R S350A	wt
MutM	S98N T127A E196K	T127A	T127A A148E	T127A	S98N	T127A	T127A A193T	wt	T127A A148E	T127A	T127A
MutS	R19K G337E	Δ*mutS*	Δ*mutS*	Δ*mutS*	wt	R19K G337E	Δ*mutS*	G337E	A3T R19K A135T E263Q G337E T384N	M1_A9*del* R19K E263Q G337E T384N	P231L G337E
MutT	E93K S106A G109D	R89S	R89S E103K D114E	R89S	wt	wt	R89S L129V	R89S	A27_R28*ins*IS26	I40V R89S L129V	wt
MutY	wt	A217S Q272H	G101S A217S A280S	A217S	V175L S217A E241D Q272L	wt	T98A A217S	A217S Q272H	A57V T98A A217S G318D	N67S T98A A217S A280S G318D	A217S Q272H
RecA	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
RecF	wt	wt	wt	E194Q	wt	A134V	wt	wt	wt	wt	wt
RecO	wt	V123A	V123A	V123A	wt	V123A	V24I	V123A	wt	V123A	wt
RecR	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
UvrA	wt	wt	T214S R619H	wt	wt	wt	F112Y	wt	F112Y D523N E928K	F112Y	wt
UvrB	wt	wt	wt	G405C	wt	wt	wt	wt	D135A L281M	D434E	wt
UvrC	wt	wt	wt	wt	N418S	wt	wt	wt	A143V	wt	wt
UvrD	L559M	wt	S719T	wt	wt	R234C	S719T	Q434K	S719T	S719T	S719T
UvrY	M1L	wt	T45A	wt	wt	wt	T45A	M1L	T45A	T45A	M1L

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Figure S1. Southern blot analysis of *mutS* genes in five selected strains with absence of *mutS* by WGS and PCR. This figure appears in colour in the online version of *JAC* and in black and white in the printed version of *JAC*.