UNIVERSIDAD DE SEVILLA

Facultad de Medicina

Departamento de Microbiología

TESIS DOCTORAL

Optimisation of the therapeutic potential of fosfomycin against *Enterobacteriaceae*: characterisation of genetic and physiological factors related to resistance and antimicrobial activity.

Trabajo realizado para alcanzar el grado de Doctor por Dña. Miriam Ortiz Padilla

Sevilla, marzo de 2023

UNIVERSIDAD DE SEVILLA
Facultad de Medicina
Departamento de Microbiología

Dr. D. Fernando Docobo Pérez, Profesor Contratado Doctor de Microbiología de la Universidad de Sevilla; y el Dr. D José Manuel Rodríguez Martínez, Profesor Titular de Microbiología de la Universidad de Sevilla,

CERTIFICAN:

Que la tesis para optar al grado de Doctor por la Universidad de Sevilla que lleva por título "Optimisation of the therapeutic potential of fosfomycin against *Enterobacteriaceae:* characterisation of genetic and physiological factors related to resistance and antimicrobial activity" ha sido realizada por Dña. Miriam Ortiz Padilla bajo nuestra supervisión, reuniendo todos los requisitos necesarios para su presentación mediante la modalidad de compendio de publicaciones.

Y para que conste donde proceda firmamos el presente certificado en Sevilla, marzo 2023.

Dr. D Fernando Docobo Pérez | Dr. D. José Manuel Rodríguez Martínez

UNIVERSIDAD DE SEVILLA
Facultad de Medicina
Departamento de Microbiología

Dr. D José Manuel Rodríguez Martínez, Profesor Titular de Microbiología de la Universidad de Sevilla,

CERTIFICA:

Que la tesis para optar al grado de Doctor por la Universidad de Sevilla que lleva por título “Optimisation of the therapeutic potential of fosfomycin against *Enterobacteriaceae*: characterisation of genetic and physiological factors related to resistance and antimicrobial activity” ha sido realizada por Dña. Miriam Ortiz Padilla bajo mi tutela y es apta para ser presentada mediante la modalidad de compendio de publicaciones.

Y para que conste donde proceda firmo el presente certificado en Sevilla, marzo 2023.

Dr. D. José Manuel Rodríguez Martínez

AGRADECIMIENTOS

Este es el resultado, mi tesis. Cuesta pensar que tantas horas de trabajo se resuman en las páginas que vienen a continuación, pero claro, aquí solo se cuenta una parte. Y como en toda historia hay mucha gente detrás de ésta.

No voy a empezar a contar desde el principio, sería muy largo, aunque desde el principio siempre han estado mi familia. Ellos han apoyado desde el primer momento que me viniera a vivir a un sitio con menos olivos, que viniera a vivir y que quisiera quedarme, acabando por entender que me gustara tanto esta ciudad. Y lo más importante, aguantando el gran repertorio de malas caras que puedo poner cuando estoy agobiada…que han sido muchas veces durante los últimos años; pero como bien dijo mi amiga Irene, estamos en estado "pretésico" y nos convertimos en personas un poco más desagradables de lo que las personas de alrededor se merecen, pero se pasa.

Llevo ya muchos años en esta ciudad, solo unos pocos menos en este laboratorio, que ha acabado por ser mi casa, y la gente, un poco mi familia también. Y como por algún lugar hay que empezar, empezaremos a agradecer a Jose Manuel, co-director de esta tesis, que me acogió en su equipo para hacer el TFG (aunque no tenía más remedio), pero ya voluntariamente, para hacer también el TFM. Durante la carrera, suponía que me gustaba la microbiología, pero estar en este laboratorio con Jose Manuel y Paula enseñándome todo lo que dio tiempo, vi que me encantaba, y que mientras la ciencia me dejara, era el camino que quería seguir. Y es que Paula me enseñó como se trabajaba en micro, desde cero, dedicando horas y horas conmigo, saliendo tarde si hacía falta. Mi mano en el laboratorio es gracias a ella, y algunas manías como que cuando una placa lleva kanamicina, se rotula en rojo.

Jose Manuel también fue el que me propuso para realizar la tesis, en este caso con Fernando, el otro co-director de esta tesis. Para agradecer todo a Fernando me faltarían páginas. Mi mano en el laboratorio es gracias a Paula, pero en este caso, mi cabeza es gracias a Fernando, porque cuando pasas horas pensando sobre la fosfomicina con alguien, al final eso te enseña a pensar. Horas pensando, resolviendo problemas, sonriendo cada vez que entraba en el laboratorio diciendo que los resultados eran raros, y sonriendo aún más cuando decían que los resultados tenían buena pinta.

También tengo que agradecer a Álvaro Pascual la oportunidad de formar parte de su grupo durante tantos años. Y por supuesto a Sofía Ballesta, siempre intentando ayudar en todo, en el trabajo y en la vida. Atendiendo con una sonrisa sin importar la de veces que entremos en su despacho.

Tiene que tener un párrafo, aunque para poner todo necesitaría un par canario de párrafos (que puede ser cualquier número par entre 2 y el infinito), y es que Sara ha acabado por ser parte de mi vida, dentro y fuera del laboratorio. Porque es la persona con la que puedo pasar todo el día en el trabajo, ir a entrenar, luego salir y cuando llego a casa, estar una hora mandándonos audios para comentar el día…

Muchas gracias al resto del "equipo fosfo", Inés y Belén, y luego Ana. Dispuestas siempre a pensar conmigo y frustrarnos juntas, y hacer todos los experimentos que se nos

ocurrían, incluso de un día para otro. Y al resto del laboratorio los de antes y los de ahora, Ana Duque y Adri, que ya no están en el lab, pero están fuera, que es más importante. A Marinilla, que igual te llama la atención y te pone a leer los protocolos originales, te da un abrazo y te cuida. A Andrea, que sigue viniendo a desayunar los viernes y con la que ojalá hubiese compartido un zulito bioinformático, lleno de fotos de gatos. A Marisa, que siempre ayuda en el lab y además, te manda fotos y videos de Mechi, siempre que es necesario, y si no también los manda, y nunca están de más. A Laurita y María, que han aguantado todo este colapso final, todo el estrés, viendo un poco lo que les espera, y animando como mejor han sabido, aunque fuera comiendo sándwich de nocilla a las 6 de la tarde. A Jose Luis, que viene y hace "ciencia de tarde", también conocida como cosas absurdas que hay que hacer (medir tubos o calibrar bombas, el Hollow-Fiber tiene esas cosas), no necesitan mucha concentración y te puedes reírte un poco mientras, porque a veces el día ya ha sido muy largo, pero aún no te puedes ir.

Y la gente de fuera del laboratorio, Irene y Maribel, que llevan conmigo desde el primer día de carrera, que no siempre tenemos muy claro qué va a ser de nosotras, pero ahí vamos…Siempre puedes mandarles una decena de audios, y tener conversaciones largas que duran días. Y por su puesto a las niñas del jiuji, que aparecieron hace nada, me acogieron y me descubrieron un maravilloso mundo, y ahora son absolutamente necesarias en mi vida…

Seguramente me dejo gente y cosas importantes que decir…Pero soy rancia jiennense, así que es complicado escribir estas cosas para mí. Así que a todas las personas que han estado ahí estos últimos años, ayudando en la tesis, o a que se me pase la frustración por la misma, de verdad, gracias.

Tesis como compendio de artículos previamente publicados.

Esta tesis, de acuerdo con el informe correspondiente y la autorización de los directores de la misma, y del órgano responsable del Programa de Doctorado, se presenta como un compendio de tres artículos. Las referencias completas a los artículos que componen el cuerpo de la tesis son las siguientes:

- Ortiz-Padilla M, Portillo-Calderón I, de Gregorio-Iaria B, Blázquez J, Rodríguez-Baño J, Pascual A, Rodríguez-Martínez JM, Docobo-Pérez F. **Interplay among Different Fosfomycin Resistance Mechanisms in *Klebsiella pneumoniae*.** Antimicrob Agents Chemother. 2021 Feb 17;65(3):e01911-20. doi: 10.1128/AAC.01911-20. PMID: 33361305; PMCID: PMC8092493.

- Ortiz-Padilla M, Portillo-Calderón I, Maldonado N, Rodríguez-Martínez J, de Gregorio-Iaria B, Merino-Bohórquez V, Rodríguez-Baño J, Pascual Á, Docobo-Pérez F. **Role of inorganic phosphate concentrations in in vitro activity of fosfomycin.** Clin Microbiol Infect. 2022 Feb;28(2):302.e1-302.e4. doi: 10.1016/j.cmi.2021.09.037. Epub 2021 Oct 8. PMID: 34634457.

- Ortiz-Padilla M, Portillo-Calderón I, Velázquez-Escudero A, Rodríguez-Baño J, Pascual Á, Rodríguez-Martínez JM, Docobo-Pérez F. **Effect of Glycerol on Fosfomycin Activity against *Escherichia coli*.** Antibiotics (Basel). 2022 Nov 12;11(11):1612. doi: 10.3390/antibiotics11111612. PMID: 36421256; PMCID: PMC9686493

I. LIST OF ABBREVIATIONS AND ACRONYMS **1**

II. ABSTRACT **3**

III. INTRODUCTION **7**

1. Bacterial resistance 7
2. The public health problem of antimicrobial resistance. 8
3. Strategies for the fight against antimicrobial resistance. 12
   - 3.1. Bacterial resistance monitoring system. 12
   - 3.2. Infection prevention. 12
   - 3.3. Optimizing the use of available antimicrobials. 13
   - 3.4. Reduction of the use of antimicrobials for non-therapeutic purposes. 13
   - 3.5. Development of new antimicrobials. 14
   - 3.6. Development of new therapeutic strategies. 15
   - 3.7. Rescue of old antimicrobials. 15
4. Fosfomycin. 16
   - 4.1. History 16
   - 4.2. Molecular characteristics. 17
   - 4.3. Mechanism of action. 17
   - 4.4. Regulation and activity of fosfomycin transporters. 18
     - Regulation of cAMP-CRP complex 18
     - Regulation of *uhpT* 20
       - Transporter structure, expression, and transporter activity 20
     - Regulation of *glpT* 21
       - Structure 21
       - Transporter expression 22
       - Glycerol-mediated induction 23
       - Transporter activity 23
     - Phosphate homeostasis 24
   - 4.5. Resistance mechanisms. 24
     - Intrinsic resistance 24
       - Mutation in the *murA* gene 24
       - Peptidoglycan recycling pathways 25
       - Other intrinsic mechanisms 25
     - Acquired resistance 25
       - Mutations in *murA* 25
       - Decreased transport of fosfomycin 25

- Intrinsic and acquired resistance mechanisms ... 26
  - Antibiotic-modifying enzymes ... 26
    - *fosB*: ... 27
    - *fosX*: ... 27
  - *fosA* ... 27
    - Sodium phosphonoformate ... 28

4.6. Spectrum of action and epidemiology ... 29
- Gram-positive spectrum of action ... 29
- Gram-negative spectrum of action ... 30
- Gram-negative epidemiology ... 31

4.7. Susceptibility studies ... 32
- Agar dilution: ... 32
- Broth microdilution: ... 33
- Disc diffusion: ... 34
- Gradient diffusion strips: ... 34

4.8. Clinical categories (Susceptibility breakpoints) ... 35
- Clinical breakpoints ... 35
- Epidemiologic Cut-Off: ECOFF ... 36
- Fosfomycin susceptibility breakpoints ... 37

4.9. Subpopulations ... 39
- Heteroresistance ... 39

IV. HYPOTHESES AND OBJECTIVES ... 42

1. Hypothesis ... 42

2. Objectives ... 42

V. PUBLICATIONS ... 43

1. Interplay among Different Fosfomycin Resistance Mechanisms in *Klebsiella pneumoniae* ... 44

Supplementary Material of Interplay among Different Fosfomycin Resistance Mechanisms in *Klebsiella pneumoniae*. ... 56

2. Effect of Glycerol on Fosfomycin Activity against Escherichia coli. ... 62

Supplementary Material of Effect of Glycerol on Fosfomycin Activity *against Escherichia coli.* ... 77

3. Role of inorganic phosphate concentrations in in vitro activity of fosfomycin. ... 81

Supplementary Material of Role of inorganic phosphate concentrations in in vitro activity of fosfomycin. ... 87

VI. DISCUSSION ... 93

VII. CONCLUSIONS ... 100

VIII. BIBLIOGRAPHY ... 101

I. LIST OF ABBREVIATIONS AND ACRONYMS

AEMPS: *Agencia española del medicamento y productos sanitarios* (Spanish Agency for Medicines and Medical Devices)

AUC: Area under the curve

cAMP: Cyclic adenosine monophosphate

CDC: Center for diseases control and prevention

CFU: colony forming units

CLSI: Clinical & Laboratory Standards Institute

Cmax: Maximum concentration

COESANT: *Comité Español del Antibiograma* (Spanish Antibiogram Committee)

COMBACTE-MAGNET: Combatting Bacterial Resistance in Europe-Molecules against Gram-Negative Infections

CRP: complex receptor of cAMP

DIN: *Deutsches Institut für Normung*

EAR: Emerging Antimicrobial Resistance Reporting

EARS-Net: European Antimicrobial Resistance Surveillance Network

ECDC: European Centre for Disease Prevention and Control

ECOFF: Epidemiological cut-off values

EC50: concentration the produce 50% of inhibition

EMA: European Medicines Agency

ENABLE: *European Gram-Negative Antibacterial Engine*

ESBL: extended-spectrum beta-lactamases

ESCMID: European Society for Clinical Microbiology and Infectious Disease

EUCAST: European Committee on Antimicrobial Susceptibility Testing

FAO: Food and Agriculture Organization of the United Nations

FDA: US Food and Drug Administration

GARDP: Global Antibiotic Research and Development Partnership

GLASS: Global Antimicrobial Resistance Surveillance System

G3P: glycerol-3-phosphate

G6P: glucose-6-phosphate

HPr: histidine protein

JPIAMR: Joint Programming Initiative on Antimicrobial Resistance

KPC: *K. pneumoniae* carbapenemase

LOD: Limit of detection

MIC: minimum inhibitory concentration

MFS: Major Facilitator superfamily

MRSA: Methicillin-resistant *S. aureus*

NCCLS: National Committee for Clinical Laboratory Standards

ND4BB: New Drugs for Bad Bugs

OECD: Organisation for Economic Co-operation and Development

OIE: Office International des Epizooties

PEP: phosphoenolpyruvate

Pi: inorganic phosphate

PIRASOA: *Programa integral de prevención y control de las infecciones relacionadas con la asistencia sanitaria y uso apropiado de los antimicrobianos* (Integrated program for the prevention and control of healthcare-associated infections and appropriate use of antimicrobials)

PD: pharmacodynamics

PK: pharmacokinetics

PPF: sodium phosphonoformate

PRAN: *Plan Nacional frente a la Resistencia a los Antibióticos* (National Plan against Antimicrobial Resistance)

PROA: *Programas de optimización de tratamientos antimicrobianos* (Antimicrobial therapy optimization programs)

PTS: phosphotransferase sugar

REPAIR: *Replenishing and Enabling the Pipeline for Anti-Infective Resistance*

SFM: *Société Française Microbiologie*

WHO: World Health Organisation

ZIP*: Zero Interaction Potency Model*

II. ABSTRACT

Since the discovery of the first antimicrobials, bacteria with resistance mechanisms against them have been detected. The appearance of bacterial resistance is a natural phenomenon, which has increased as a result of the use of antimicrobials. Therefore, the availability of antimicrobials does not ensure therapeutic success. Moreover, in recent decades, a progressive increase in antimicrobial resistance has occurred, and it has become a global public health problem, since there is an increase in deaths caused by or related to bacteria that present resistance mechanisms. As a result of this problem and the scarcity of new effective molecules for the treatment of multidrug-resistant bacteria, various organizations (such as WHO and FAO) are developing plans with different strategies to address the problem. These strategies include optimizing of the use of existing antimicrobials and the rescue of old antibiotics that are still active, such as fosfomycin.

Fosfomycin is an old antimicrobial that can be a good therapeutic option, since it many bacteria of clinical interest remain sensitive to this antibiotic. Fosfomycin is a derivative of phosphonic acid, a hydrophilic, low molecular weight molecule. It has three carbon atoms, is soluble in water, and is similar to phosphoenolpyruvate. It is a broad-spectrum bactericidal antimicrobial that acts in the growth phase of bacteria, inhibiting the first step of cell wall peptidoglycan synthesis by binding to the enzyme MurA. Fosfomycin must penetrate the cytoplasm to reach its target, MurA, producing bactericidal effect. For this purpose, two membrane transporters GlpT and UhpT are described, whose physiological function in bacteria is the uptake of phosphorylated carbon sources and expel inorganic phosphate (Pi). The regulation and activity of these transporters is fundamental to the mechanism of action of fosfomycin and, therefore, to fosfomycin resistance.

The transcription of both transporters is induced by their own substrate, in addition to the AMPc-CRP metabolism regulator complex, and they are also activated by the FNR regulator, a bacterial regulator under anaerobic conditions. The GlpT transporter has the function of introducing glycerol-3-phosphate (G3P), this molecule binds to the GlpR repressor, causing the loss of affinity for promoters of the *glp* regulon genes, such as *glpT*. On the other hand, UhpT transports hexose-phosphate, mainly glucose-6-phosphate (G6P). This molecule is detected by a two-component system, UhpB and C, and when this occurs, it phosphorylates UhpA, which binds to the *uhpT* promoter, inducing its transcription. Thus, in the susceptibility assays G6P must be added to induce the presence of this transporter, as the susceptibility results obtained in this way are more consistent with susceptibility breakpoints and therapeutic success.

Fosfomycin resistance mechanisms can be plasmid and chromosomal mediated, as modifications of MurA, the presence of peptidoglycan recycling pathways, alteration of fosfomycin

permeability or the presence of fosfomycin-modifying enzymes. Chromosomal mediated fosfomycin resistance usually occurs in a stepwise mode, often generating complex phenotypes difficult to interpret.

In this sense, to better understand the mechanisms of resistance to fosfomycin in *Klebsiella pneumoniae* and to optimize the use of this antimicrobial, the following study was carried out. The objectives were to characterize the role of the genes *uhpT*, *glpT*, and *fosA* in resistance to fosfomycin in *K. pneumoniae* and to evaluate the use of phosphonoformate sodium (PPF) due to its ability to inhibit the FosA enzyme, in combination with fosfomycin. For this purpose, seven clinical isolates of *K. pneumoniae* and the reference strain (ATCC 700721) were used, and their genomes were sequenced. Mutants for transporters and *fosA* were constructed from two isolates of *K. pneumoniae* ATCC 700721. The susceptibility test to fosfomycin was performed using the gradient strip method. Synergy between fosfomycin and PPF was studied by checkerboard assay and analyzed with SynergyFinder. Spontaneous frequencies of occurrence of fosfomycin and PPF mutants, *in vitro* activity by growth curves with gradient concentrations of fosfomycin with and without PPF, and time-kill assays with and without PPF were also evaluated. The fosfomycin MICs of the clinical isolates ranged from 16 to 1,024mg/L. The addition of 0.623 mM PPF reduced the MIC by 2 to 8-times. Deletion of *fosA* gene led to a 32-fold decrease. Synergistic activities were observed with the combination of fosfomycin and PPF (most synergistic area at 0.623mM). The lowest frequencies of fosfomycin resistant mutants were found in Δ*fosA* mutants with frequency ranging from 1.69×10^{-1} to 1.60×10^{-5} for 64 mg/L fosfomycin. Finally, the growth monitoring and time-kill assays, fosfomycin showed bactericidal activity only against *fosA* mutants and not with the addition of PPF. **The study concludes that inactivation of the *fosA* gene results in decreased resistance to fosfomycin in *K. pneumoniae*. The pharmacological approach using PPF did not achieve sufficient activity and the effect decreased with the presence of other fosfomycin resistant mutations.**

The second chapter of the Thesis follows the line of optimizing the use of fosfomycin with the addition of an adjuvant, and to better understand how the regulation of fosfomycin transporters may affect their activity. The main objective was to evaluate the role of glycerol at therapeutically relevant concentrations in combination with fosfomycin in *Escherichia coli*, since this molecule is clinically used as a treatment for example for elevated intracranial pressure and can induce *glpT* expression. For this purpose, a collection of isogenic mutants of fosfomycin-related genes was evaluated in *E. coli* strains. The induction of fosfomycin transporters was evaluated and susceptibility tests, interaction assays, and time-to-death assays were performed. Our results showed that glycerol allows the activation of the GlpT transporter, but this induction is delayed in time and is not homogeneous in all *E. coli* strains throughout the bacterial population, leading to contradictory results in terms of fosfomycin activity.

The susceptibility assays showed increased fosfomycin activity with glycerol in the disc diffusion assay, but not in the agar dilution or broth microdilution assays. Similarly, in time-kill assays, the effect of glycerol was absent because of the appearance of fosfomycin-resistant subpopulations. **In conclusion, glycerol may not be a good candidate for use as an adjuvant to fosfomycin.**

Finally, to better understand physiological factors that affect fosfomycin transporters activity, the aim of third chapter was to evaluate the *in vitro* activity of fosfomycin under different physiological concentrations of inorganic phosphate (Pi). For this purpose, the wild-type strain BW25113, four isogenic mutants (Δ*glpT*, Δ*uhpT*, Δ*glpT-uhpT* and Δ*phoB*) and six clinical isolates of *E. coli* with different fosfomycin susceptibilities were used. Susceptibility was assessed by agar dilution using Mueller-Hinton agar (Pi=1mM) and supplemented with Pi (13 and 42mM, minimum and maximum urinary concentrations of Pi) and/or glucose-6-phosphate (25mg/L). The promoter activity of the fosfomycin transporter was assessed by monitoring fluorescence accumulation using pUA66-P*glpT*::*gfp*mut2 or pUA66-P*uhpT*::*gfp*mut2 plasmids in standard Mueller-Hinton broth (MHB) supplemented with Pi (13 or 42mM) ± glucose-6-phosphate. Fosfomycin activity was quantified spectrophotometrically at 24 hours as before with glucose-6-phosphate, and fosfomycin ranged from 1 to 1024mg/L. The EC50 of fosfomycin was estimated and compared. Time-kill assays were performed with fosfomycin concentrations of 307 (plasma Cmax), 1053 and 4415mg/L (urinary Cmax range), using MHB with 28mM Pi (mean urinary concentration) +25mg/L glucose-6-phosphate. The results showed that all strains decreased fosfomycin susceptibility linked to increasing Pi concentrations: 1-4-log2 dilution differences from 1 to 13mM, and 1-8-log2 dilution differences at 42 mM Pi. Changes in phosphate concentration did not affect the expression of fosfomycin transporter promoters. Also, increasing Pi concentrations resulted in a higher bacterial viability EC50 of fosfomycin, except against the Δ*glpT-uhpT* mutant strain. **Therefore, the present study concludes that Pi variations in physiological fluids may reduce the activity of fosfomycin against *E. coli*. Also, the elevated urinary Pi concentrations may explain the failure of oral fosfomycin in non-wild but fosfomycin-susceptible *E. coli* strains.**

III. INTRODUCTION

1. Bacterial resistance

The development of antimicrobials has been one of the greatest advances in the field of health since it has made it possible to cure numerous infectious processes[1]. After the discovery of penicillin and its possible uses in the 1940s by Alexander Fleming, he himself warned in 1945 that the availability of antimicrobials did not ensure therapeutic success, since microorganisms adapted, becoming resistant to treatment and, therefore, reducing their efficacy[2]. The emergence of bacterial resistance is a natural phenomenon that is inherent in microorganisms, and efforts have been made to overcome it with the introduction of new antimicrobials. Despite this, soon after their use, microorganisms with resistance mechanisms against them appear[1,3].

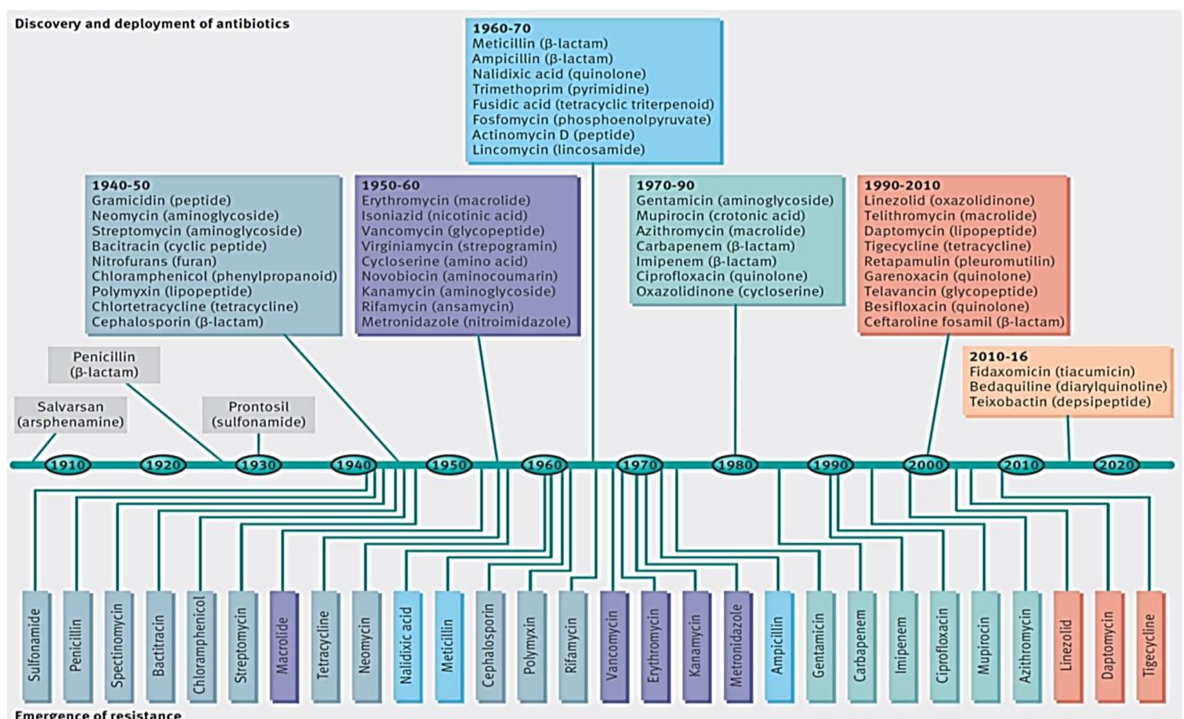


Figure 1. Timeline showing the introduction of antibiotics into clinical practice and the emergence of antimicrobial resistance. Most antibiotic scaffolds were discovered up to the 1970s. Then, most of them were chemically expanded by introducing new functional groups[4].

The beginning of the study of bacterial resistance occurred when the decrease in the efficacy of antimicrobial treatments began to be detected, although the existence of resistance mechanisms predates the use of these substances[1,5,6]. The existence of these factors, prior to the introduction of antimicrobial treatments, has been demonstrated in some studies and can be explained by the evolution of microorganisms coexisting in the same natural habitat, accumulating protective and metabolic mechanisms that promote their survival[7–9]. The set of genes involved in antimicrobial resistance that bacteria have been acquiring is called the "antimicrobial resistome", a term that began to be used in 2006 by Gerry Wright's group in an article on the determinants of resistance present in

soil[10]. Subsequently, the resistome was defined as the set of all genes conferring resistance to antimicrobials and precursors, in pathogenic and non-pathogenic bacteria. This includes all types of resistance genes, both acquired and intrinsic, their precursors, and some potential resistance mechanisms in microbial communities that require evolution or altered gene expression to confer resistance. The study of resistance since then, based on the resistome concept, has confirmed that; (i) antimicrobial resistance is ancient and ubiquitous in microbiomes, (ii) the resistome is complex and diverse, (iii) the environmental resistome is the origin and reservoir of antimicrobial resistance genes, (iv) the resistome is determined by microbial community structure in the natural environment, (v) human activities modify the environmental resistome, (vi) mobile genetic elements are responsible for the transmission of these genes, and (vii) antimicrobial resistance genes flow between humans, animals, and the environment. These findings have served as a basis for approaching a solution to the resistance problem from a "One-Health" point of view, that the study of antimicrobial resistance should be directed at all sectors: human, animal, and environmental, as all are affected by each other[10,11].

In general, antimicrobial resistance mechanisms can be summarized as: (i) genes captured by gene mobilization and gene transfer (conjugation, transduction, and transformation); (ii) chromosomal mutations, which usually have a biological cost, but can be accompanied by compensatory mutations; (iii) overexpression of intrinsic resistance mechanisms, such as enzymes that inactivate antimicrobial, or efflux pumps[12–14].

This natural phenomenon has been amplified by the extensive use of antimicrobials by humans, both in medicine and veterinary medicine, as well as their disposal as waste. This has helped the evolution and diffusion of resistance mechanisms both in clinical environments and in natural ecosystems. All this has led to the spread of bacteria resistant to different antimicrobials simultaneously (multidrug-resistant bacteria), especially in clinical settings, although they are increasingly distributed in community environments, making the treatment of infections caused by these pathogens more difficult, since they allow few therapeutic options[15–17]. These infections are associated with increased mortality and hospital stay, and therefore also increase the economic cost. Therefore, antimicrobial resistance has been identified as a threat to public health and the global economy[18].

2. The public health problem of antimicrobial resistance.

In the last decade, different studies on infections caused by microorganisms carrying antimicrobial resistance mechanisms have shown the increase in morbidity, mortality, and therefore the increase in the economic cost of infection[19,20].

The massive use of antimicrobials, both in health care and in industry (mainly livestock) causes the selection and evolution of resistance mechanisms in microorganisms. On the other hand, globalization throughout the world has facilitated the mobility of these microorganisms, leading to an exponential increase in pathogens resistant to existing treatments[21,22].

Genes encoding resistance mechanisms can be inherited, but they can also be transmitted between bacterial species by mobile genetic elements such as integrons, transposons, or plasmids. In addition, there are what are known as "successful" or "high-risk" clones. These bacterial clones have great relevance in the dispersion of resistance mechanisms because they accumulate and exchange resistance mechanisms effectively, have a high pathogenic, invasive and colonizing capacity, and therefore also have high transmissibility between people.[22,23].

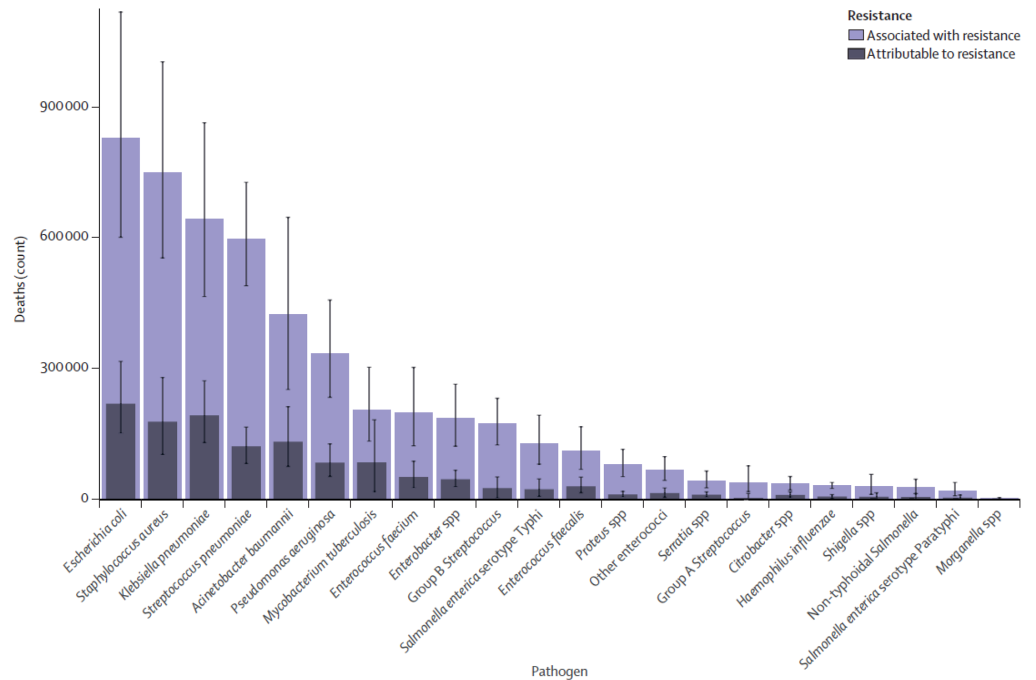


Figure 2. Global deaths (counts) attributable to and associated with bacterial antimicrobial resistance by infectious syndrome, 2019[24].

All of this is of great epidemiological interest and has been the subject of study. The first review of the global health and economic consequences of bacterial resistance was published in 2014 by economist Jim O'Neil. This review estimates that 700,000 people die each year from infections caused by antimicrobial resistant pathogens and estimates that by 2050 there will be 10 million deaths, making it the leading cause of death worldwide, if the trend of increasing and expanding bacterial resistance continues[18]. Another major study conducted in 2019, which extracted data on 23 pathogens in 204 territories, showed results on AMR-associated deaths. Estimated, through statistical predictive modelling, there were 4.95 million (3.62-6.57) deaths associated with AMR, including 1.27 million (0.911-1.71) deaths attributable to AMR in 2019. The six leading pathogens for AMR-associated deaths

were *Escherichia coli*, followed by *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*. These pathogens were responsible for 929000 (660 000-1270 000) AMR-attributable deaths attributable to AMR and 3.57 million (2.62-4.78) deaths associated with AMR in 2019[25].

The main cause of the increase in bacterial resistance is the consumption of antimicrobials[26], which has been increasing globally, raising by 65% between 2000 and 2015; and if the trend continues, it will increase by around 200% in the next 10 years[27]. However, antimicrobial consumption and bacterial resistance are not evenly distributed throughout the world. An increase in antimicrobial resistance can be observed in countries with lower socioeconomic and health inequities levels[27].

This public health problem has managed to become one of the main points on the international agenda, in which various institutions such as the World Health Organization (WHO), the Food and Agriculture Organization of the United Nations, or better known as FAO, and the World Organization for Animal Health (Office International des Epizooties or OIE) have prepared documents proposing solutions to alleviate this threat to public health. In 2015, the WHO approved the Global Action Plan on Antimicrobial Resistance. This document recognizes the magnitude of the problem of antimicrobial resistance worldwide and proposes measures to be carried out, all from a One-Health perspective, i.e., medicine, veterinary medicine, the environment, agriculture, and society in general should be involved[11,21]. The general measures proposed are: (i) improving awareness of antimicrobial resistance and behavioural change among policy makers, farmers, veterinarians, and health personnel, the food industry and the general public through education, communication and training on the most efficient topic; (ii) strengthening the knowledge and evidence base through research and surveillance; (iii) reducing the incidence of infections in healthcare facilities, farms, and communities, as well as environmental contamination, through effective prevention; (iv) optimizing the use of antimicrobials in human and animal health; eliminating the use of animals for growth promotion; (v) increasing the development of R&D development on new drugs, diagnostics, vaccines, and other interventions related to priority pathogens[21]. The ultimate objectives of this plan are to maintain reduced levels and decrease the development of resistance in order to maintain the ability to treat infectious diseases with effective and safe drugs. All this would reduce the impact of infectious diseases in humans and animals at the health and economic levels. This is being monitored and evaluated at different levels; regionally, nationally, and globally. FAO, OIE and WHO are individually monitoring activities and results against each organization's plan and budget, collectively tracking progress and reporting on joint work plan activities. The Inter-Agency Coordination Group on Antimicrobial Resistance may recommend additional issues and indicators for monitoring[28].

In the European framework, in the report published by the European Antimicrobial Resistance Surveillance Network (EARS-Net) in 2021[25], a network that collects and analyzes bacterial resistance in 29 countries of the European Economic Area, data were reported for eight pathogens*: Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter spp*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Enterococcus faecalis*, and *Enterococcus faecium*. The most reported bacterial species was *E. coli* (39.4%), followed by *S. aureus* (22.1%), *K. pneumoniae* (11.9%), *E. faecalis* (8.8%), *E. faecium* (6.2%), *P. aeruginosa* (6.1%), *Acinetobacter spp* (3.0%) and *S. pneumoniae* (2.5%). In 2021, the overall number of reported isolates increased compared to 2020 for all bacterial species. The reported status of antimicrobial resistance varied widely depending on bacterial species, antimicrobial group, and geographic region. In general, most bacterial species/antimicrobial combinations in this report showed a significantly decreasing trend or no significant trend during 2017-2021. The exceptions were resistance to carbapenem in *E. coli* and *K. pneumoniae* and resistance to vancomycin in *E. faecium*, for which there was a significant increase during this period. Importantly, in 2021, more than half of the reported *E. coli* strains and more than one-third of *K. pneumoniae* strains were resistant to at least one antimicrobial group, and multidrug resistance (combined resistance to several antimicrobial groups) was a frequent occurrence. Resistance rates were generally higher in *K. pneumoniae* than in *E. coli*, as in the case of resistance to carbapenems, being higher in *K. pneumoniae*, but not frequently found in *E. coli*[25]. Resistance patterns are not homogeneous; generally, a higher rate of resistance is observed in countries located further south and east of the European economic area[25].

If we focus on our country during 2020, antimicrobial sensitivity data corresponding to a total of 20,438 isolates of blood cultures and cerebrospinal fluids from the same number of patients have been analyzed, which represents an increase of 6.7% compared to 2019. In the report communicated by EARS-Net, several points can be highlighted. Resistance to all antimicrobials studied in *E. coli* has increased from 2001 to 2020, and a quarter of strains show resistance to three or bad families of antibiotics. In 10 years, resistance to third generation cephalosporins has increased from 10.2% to 30%, an increase that has been accompanied by other antimicrobials such as fluoroquinolones (30%) and aminoglycosides (17%). Resistance to carbapenems has reached the highest figures, reaching 0.2% for *E. coli* and 4.7% for *K. pneumoniae*. Methicillin-resistant strains of *S. aureus* remain stable and the trend of a slight decrease in resistance in general and beta-lactams in particular has been maintained[29].

Therefore, antimicrobial resistance is a public health problem that also affects global ecology. For this reason, different international organizations are proposing surveillance and analysis to promote different strategies to solve the problem.

3. Strategies for the fight against antimicrobial resistance.

The discovery and use of antimicrobials for prophylaxis and treatment have led to great medical advances. But, at the same time, the problem of bacterial resistance was growing and spreading globally, threatening the usefulness of these drugs. For this reason, it became necessary to take measures to control the emergence and spread of resistance mechanisms. These measures must be taken with a One Health approach, that is, in a multidisciplinary way, working together to try to solve or alleviate this problem that concerns human, animal, and environmental health[11,30]. To this end, in recent years, different organizations have proposed national and international programs whose main measures would be the following[31]:

3.1. Bacterial resistance monitoring system.

The control of antimicrobial resistance requires surveillance, monitoring, and communication of data obtained on resistant microorganisms at the global, international, and national levels. To this end, programs such as the Global Antimicrobial Resistance Surveillance System (GLASS), which collects, analyzes, and exchanges resistance data worldwide[21], the aforementioned EARS-Net at the European level[32], or the National Plan against Antimicrobial Resistance (PRAN) have been established. The PRAN was approved in Spain in 2014 in which all autonomous communities, different ministries, as well as scientific societies, scientific organizations, and expert collaborators work together[33]. These plans allow tracking, study of resistance distribution, and analysis of the data obtained to develop specific interventions[31].

3.2. Infection prevention.

The aim of infection prevention is to reduce the spread of microorganisms in order to avoid or reduce the spread of resistance mechanisms; at the same time, the reduction of infections would allow a reduction in the use of antimicrobials[34]. In the hospital setting, prevention measures have been shown to have a real impact on reducing bacterial transmission. Measures such as cleaning and decontamination of surfaces or hand washing; in addition to screening and isolation systems for patients carrying microorganisms with resistance mechanisms[35].

Education of the population in basic hygiene measures, in addition to a good urban waste management system, is fundamental to preventing the transmission of infectious diseases[36,37]. Another important point is environmental cleanliness, with waste treatment in general, and also with water control, with greater emphasis on hospital wastewater where both, microorganisms and antimicrobials, have been found that would facilitate the selection of resistance mechanisms[38].

3.3. Optimizing the use of available antimicrobials.

Inappropriate use of antimicrobials promotes an increase in resistance to these drugs. It was estimated that, in humans, more than half of the treatments are not appropriate[39]. To avoid unnecessary, inappropriate, or excessive treatment, it is necessary to know the microbiological epidemiology and up-to-date training to select the most appropriate antimicrobial treatment and dosage. To achieve the optimized use of antimicrobials, PROA programs are implemented[40]; coordinated by physicians specializing in infectious diseases, microbiologists, pharmacists, epidemiologists, nurses, and other specialists in the affected areas; they aim to optimize treatments, reduce costs, and avoid the emergence of bacterial resistance[41]. The implementation of these programs has improved the control of bacterial resistance, reduced healthcare-associated infections, and associated hospital costs[42], and could save some 4.8 billion dollars a year and up to 1.6 million lives by the year 2050, according to estimates by the OECD (Organisation for Economic Cooperation and Development) together with the CDC (Centers for Disease Control and Prevention) [43].

In Spain, experts from scientific societies have established national consensus on how to implement PROA programs at the hospital level[40]. In Andalusia specifically, in 2013, the Comprehensive Program for the Prevention and Control of Healthcare-Related Infections and the Appropriate Use of Antimicrobials, or also known as the PIRASOA program, was approved[44].

3.4. Reduction of the use of antimicrobials for non-therapeutic purposes.

Antimicrobials have been used since their discovery in the veterinary field, and although there are antimicrobials exclusively for that use, most are the same or share similar targets as those used for humans[45]. In addition, in veterinary medicine, antimicrobials have been used for nontherapeutic or prophylactic purposes, since they were found to serve as a dietary supplement to promote growth[46]. This led to the expansion of the use of antimicrobials in the livestock industry for this purpose[47].

Numerous studies agree that the use of antimicrobials in food production increases the development of bacterial resistance, endangering its use as a therapeutic treatment, and this has a great ecological impact[48]. Furthermore, the transmission of the resistance generated and the effect on humans is because animals act as reservoirs for microorganisms that carry resistance mechanisms. Humans come into contact with food, animals, or environmental sources such as water and can acquire the microorganisms in all these ways[49].

The regulations on the use of antimicrobials as growth promoters varies among the different countries. In Europe, it was banned in 2006, decreasing the sales of antimicrobials for veterinary use by 32.5% in ten years. Additionally, it is intended to restrict the use of some antimicrobials for prophylaxis and medicated feed[50]. But, for example, China has one of the highest consumptions of antimicrobials

destined for the food industry, has so far only banned the use of some specific drugs such as colistin, and intends to eliminate the use for this purpose by 2050[51]. This is related to the fact that one of the regions with the most multidrug-resistant strains is Southeast Asia [52].

Despite measures being taken, in 2013, antimicrobial consumption in animals in the food industry was estimated at 131,109 tons and is expected to increase to 200,000 tons by 2030[53]. To try to avoid this excessive consumption, global measures are being proposed for the regulation of the maximum consumption of antimicrobials per year, trade limits, or the introduction of taxes when used for these purposes. In addition, the WHO is proposing a guide on the use of antibiotics in food-producing animals[54], together with the FAO, which proposes programs to optimize the use of antimicrobials in livestock and agriculture, in order to reduce the impact on bacterial resistance[55].

3.5. Development of new antimicrobials.

With the increase in multidrug resistant infections, the development of new antimicrobials is necessary. Derivatives of classical antibiotics as well as novel natural and synthetic compounds are being investigated with innovative pharmaceutical development platforms[56].

There are several international initiatives and organizations supporting research and development in this field. One example of an initiative is ND4BB (Drugs for Bad Bugs), which has projects such as COMBACTE-MAGNET (Combatting Bacterial Resistance in Europe-Molecules against Gram-Negative Infections) or ENABLE (European Gram-Negative Antibacterial Engine), or the JPIAMR (Joint Programming Initiative on Antimicrobial Resistance) projects; initiatives financed by the European Union. Organizations such as REPAIR (Replenishing and Enabling the Pipeline for Anti-Infective Resistance), or GARDP (Global Antibiotic Research and Development Partnership) stand out[57].

The WHO compiled a list of priority pathogens for which research and development of new treatments against infections caused by them should be more focused. Since 2017, eight antimicrobials have been approved, although only two of them are not derived from classical antibiotic classes[58]. The problem with these types of drugs is that since they belong to classes of antimicrobials already in use, they coincide in many mechanisms of action, so there is a higher probability of the generation of resistance against them[59].

Pew Charitable Trusts is one of the sources of information that reviews antimicrobial research and development initiatives and programs. This database analyzes antibiotics in development with an emphasis on WHO-declared priority pathogens. In the 2020 review, there were 41 antibiotics in development, of which 15 were in Phase 1, 12 in Phase 3, 13 in Phase 3 and 1 had passed all trials[60].

Despite the existence of initiatives that promote the development of new antimicrobials, it is a complicated plan mainly for economic reasons. However, advances in multidisciplinary areas like bioinformatics, molecular biology, high throughput techniques, etc. are speeding up the search for molecules that may be candidates for use as antimicrobials[61].

3.6. Development of new therapeutic strategies.

The scarce development of new antimicrobials, together with the high probability of rapid generation of resistance to them, makes it appropriate to search for new nonantibiotic therapeutic strategies. These alternatives can be used as adjuvants to new or existing antimicrobial treatments, to improve their therapeutic effect; or as monotherapy treatments if present any effect by themselves[62].

Nonantibiotic substances have been developed to bind to bacterial targets, other than antimicrobials, can act on the bacteria, their environment or immunomodulators for the host undergoing infection. These molecules acting on the bacteria can inhibit resistance mechanisms, virulence factors, or bacterial physiology to enhance the action of antimicrobials[63–67]. Currently, research is being develop on the use of nonantimicrobial drugs that can have an antibiotic effect, such as anticancer drugs or antihistamines, for example[68].

Another important line of research focuses on the use of other microorganisms (probiotics or the use of bacteriophages) that can displace or kill pathogenic bacteria [69,70].

Among the strategies that enhance antimicrobial action by inhibiting resistance mechanisms, are widely known molecules such as beta-lactamase inhibitors[64], with a special focus on carbapenemase inhibitors[71], but other approaches include molecules that can inhibit enzymes such as FosA (which prevents the action of fosfomycin) [72], drugs that increase the permeability of the bacteria[73], or molecules aimed at inhibiting expulsion pumps[74].

Furthermore, inhibitors of virulence mechanisms are being developed, with a mechanism of action acting directly against these virulence factors[68] or inhibiting interbacterial communication, quorum sensing[75], responsible for the stimulation of virulence mechanisms. In addition, metabolic and physiological changes in bacteria can modulate bacterial resistance[76,77]. Modifying bacterial metabolism, inducing the translation of certain proteins, causing an increase in reactive oxygen species, or inhibiting DNA repair mechanisms can lead to cellular stress followed by bacterial death[67,77–79].

3.7. Rescue of old antimicrobials.

The development of new antimicrobials may be insufficient to alleviate the problem of treating infections caused by resistant microorganisms, thus it is possible to rescue and use old antibiotics as a therapeutic alternative because many of these molecules are still effective against many multidrug

resistant bacteria[80]. These molecules, which include antimicrobials such as chloramphenicol, minocycline, colistin, tigecycline, temocillin, nitrofurantoin, amikacin (and other aminoglycosides) or fosfomycin, were developed in the first decades of antimicrobial development, but ceased to be used due to the appearance of new, more active, and/or less toxic compounds[81].

Many of these molecules have a broad-spectrum activity and are relatively inexpensive but have not been adequately investigated. Thus, more studies are needed to optimise therapeutic dosing through novel pharmacokinetic and pharmacodynamic studies, drug-drug interactions as well as the resistance mechanisms that may emerge against them as their use increases.[80,82].

For all these reasons, the use of these old antimicrobials, should be evaluated as valid therapeutic options, in order to optimize their use and achieve the most effective treatment with the least ecological impact[82,83]. This assessment should be performed not only for the treatment of infections caused by multidrug resistant bacteria but also[84], for uncomplicated infections to protect the emergence and dissemination of resistant bacteria against the new antimicrobial treatment options.[85,86].

4. Fosfomycin

4.1. History

Fosfomycin is a natural antimicrobial derived from phosphonic acid[87]. It was discovered in Spain in 1969 using fermentation broths of the actinobacterium *Streptomyces fradiae.* Its discovery was published by the Spanish Company of Penicillin and Antibiotics, in collaboration with the Merck & Co Inc. company. Later, this molecule has been isolated from other microorganisms such as *Streptomyces viridochromogenes* and *Streptomyces wedmorensis*, *Pseudomonas syringae*, *Pseudomonas viridiflava*, and *Pseudomonas fluorescens*[88–90]; in spite of this, its current production is carried out synthetically[91].

In 1974 fosfomycin mechanism of action was described [92] and in 1983 the *in vitro* susceptibility study method was standardized[93], in which it was determined that the addition of glucose-6-phosphate (G6P) was necessary to agree with the results observed in clinical practice [94].

Originally, it was formulated for intravenous treatment, in the form of a water-soluble disodium salt and for oral treatment, in the form of a calcium salt. Subsequently, another form of oral treatment was developed, as fosfomycin-trometamol, which improves the bioavailability of the previous oral formulation and is currently the treatment of choice for this form of administration[95].

In Europe and the United States, it is recommended in its oral formulation for the treatment of uncomplicated urinary tract infections, or in some countries, as prophylaxis prior to urinary tract surgery. In its intravenous form, the use of fosfomycin use has increased for the treatment of severe

infections caused by multidrug resistant bacteria susceptible to fosfomycin. Due to this increase, different regulatory agencies such as the EMA or the FDA are reviewing its use, indications, and forms of administration[96,97]. The EMA published in 2020 recommendations for the correct use of intravenous fosfomycin as second-line treatment (when first-line treatment cannot be used or has failed) in complicated urinary tract infections, pneumonia, endocarditis or meningitis[97]. However, in the United States, fosfomycin is only approved for the treatment of urinary tract infections [98].

In the veterinary field, fosfomycin is approved for use only in Central and South America, while its use is not widespread in Europe[99].

4.2. Molecular characteristics.

Fosfomycin is a polar organophosphorus compound (cis-1,2-epoxypropyl phosphonic acid) with a low molecular weight (138 Da) and soluble in water. It has several chemical characteristics that are not usually present in organophosphorus compounds: (i) an epoxy group is attached to a negatively charged phosphorus group (this bond causes the bactericidal power of the compound), and (ii) it has a bond, without an oxygen bridge, between phosphorus and carbon. The optimal activity occurs at pH 6.2, although it is stable at pH between 4 and 11[100,101].

It is chemically unrelated to other antimicrobials, being the only representative of its own class of antibiotics. The formulation for parenteral use substitutes two hydrogens of the phosphorus radical for sodium. For oral forms, the calcium form is obtained in the same way as the sodium form, but substituting the disodium for the calcium salt; on the other hand, fosfomycin-trometamol is obtained by the addition of a tris-hydroxymethyl-aminomethane base (trometamol)[101].

Figure 3. Molecular structure of fosfomycin (A), disodium (B), calcium (C) and fosfomycin-trometamol (D)[101].

4.3. Mechanism of action.

Fosfomycin is a bactericidal antimicrobial drug whose clinical properties are based on its chemical structure and unique mechanism of action. This antibiotic inhibits the first step of bacterial cell wall peptidoglycan synthesis[102,103]. Fosfomycin irreversibly inhibits the MurA enzyme by competing with its substrate, phosphoenolpyruvate (PEP), and disrupting its binding to UDP-N-acetylglucosamine. This antimicrobial is able to covalently bind to cysteine 115 of the MurA active site by thioether bonding with its phosphonate group and also interacts electrostatically with other MurA residues (lysine 22, arginine 120 and arginine 397)[104–106]. Therefore, this antimicrobial acts in the bacterial growth phase, when wall synthesis occurs[107], and is considered a broad-spectrum antimicrobial, since its mechanism

of action allows it to have a spectrum of action that covers different Gram-positive and negative bacterial species[108].

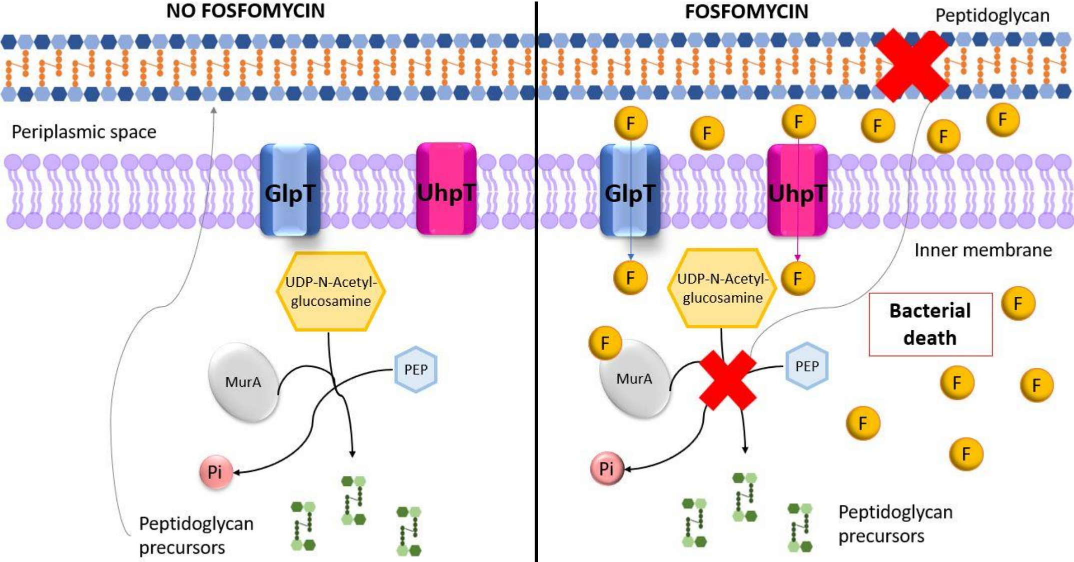


Figure 4. Mechanism of action of fosfomycin (F). NO FOSFOMYCIN) MurA synthesizes peptidoglycan precursors for bacterial growth. FOSFOMYCIN) Fosfomycin penetrates the cytoplasm through GlpT and UhpT transporters, binds to MurA, and inhibits the synthesis of peptidoglycan precursors, leading to bacterial death. Figure adapted from *Castañeda et al*[107].

Fosfomycin must penetrate into the cytoplasm, which takes advantage of two membrane transporters that are part of the major facilitator superfamily (MFS) [109]. The function of these transporters is the antiport of phosphorylated carbon sources and inorganic phosphate (Pi). UhpT is responsible for the transport of hexose-phosphate, mainly G6P and GlpT has glycerol-3-phosphate (G3P) as its main substrate[107,110]. Therefore, the regulation and activity of the transporters is essential for fosfomycin activity.

4.4. Regulation and activity of fosfomycin transporters.

The regulation and activity of these transporters is complex, as it depends on many physiological factors that are related with the bacterial metabolism, since they are transporters of carbon sources, and participate in the phosphate homeostasis.

- **Regulation of cAMP-CRP complex**

One of the main regulators of the bacterial metabolism is the dual cAMP-CRP transcriptional regulator. This complex regulates the expression of more than 180 genes[111]. It is composed of cAMP, which is synthesized by adenylate cyclase encoded by the *cyaA* gene, and CRP, which is the cAMP receptor[112,113]. Most of the regulated genes are involved in the catabolism of secondary carbon sources, but it also participates in other processes such as biofilm formation or virulence among others[114]. Generally, increasing cAMP within the bacterium increases the formation of the cAMP-CRP complex,

which induces and regulates a large number of genes that are translated into metabolic enzymes and transporters of secondary carbon sources[113], such as GlpT and UhpT[115].

This occur because the promoters of the *glpT* and *uhpT* genes present a binding site for the cAMP-CRP complex, so the increased expression of this complex would lead to a greater presence of transporters and therefore to increased entry of fosfomycin into the bacterium[115].

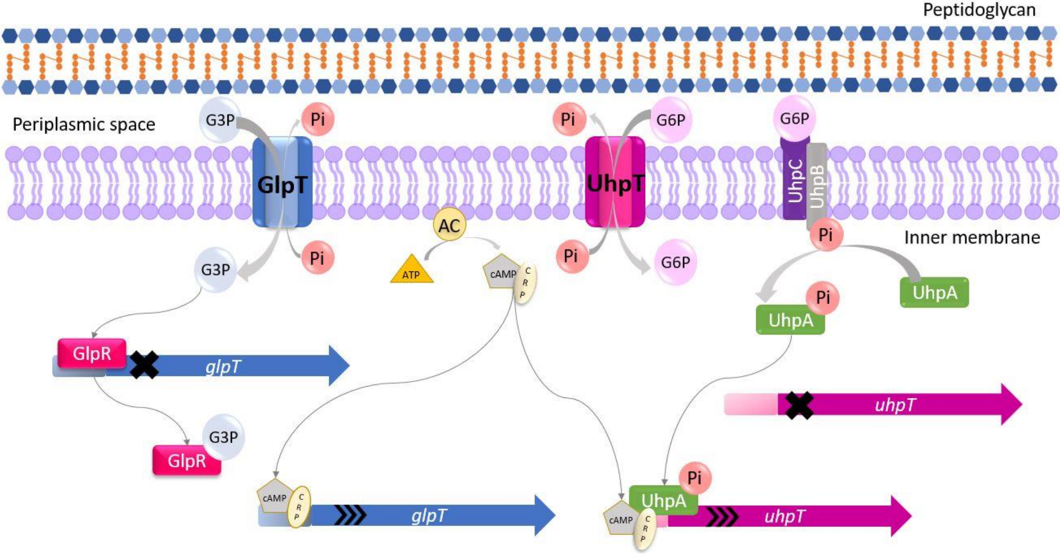


Figure 5. Representation of the transcriptional regulation system of GlpT and UhpT transporters. Figure adapted from Castañeda *et a*[107].

The regulation of the cAMP concentrations is regulated by the phosphoenolpyruvate:sugar phosphotransferase system (PTS). When the bacterium can consume glucose or another sugar incorporated by the PTS system, cellular cAMP levels decrease. The PTS system has several subunits; the *ptsI* gene encodes the first subunit, the EI protein. This protein captures a phosphate group from phosphoenolpyruvate (PEP) and phosphorylates the next subunit (HPr), which in turn phosphorylates the EIIAGlc protein. This phosphate is yielded to glucose, producing G6P, or to the PTS sugar transported. If these sugars are not present, the phosphorylated EIIAGlc protein cannot produce the phosphate group, thus inducing the expression of the *cyaA* gene, which encodes

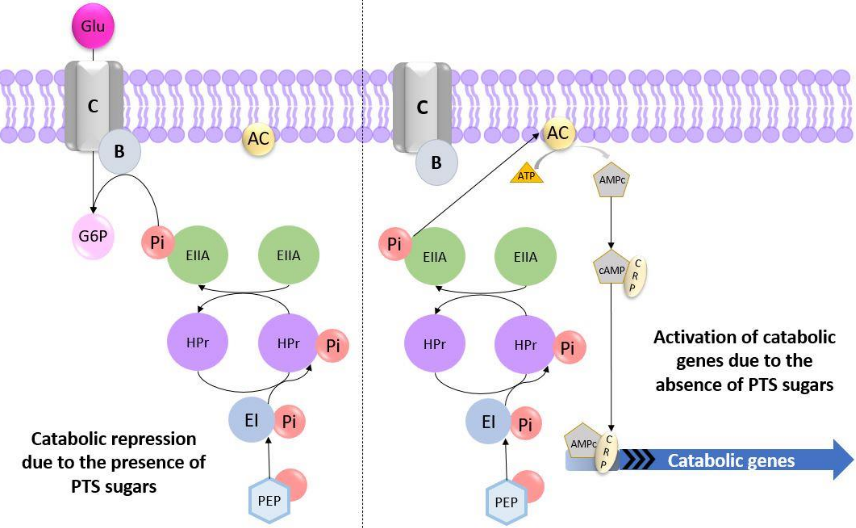


Figure 6. Representation of the cAMP-CRP complex formation system. Figure adapted from Görke *et al*[116].

adenylate cyclase (AC), the enzyme that produces cAMP[116,117]. Therefore, PTS sugars cause catabolic repression by decreasing cAMP, negatively affecting the expression of *glpT* and *uhpT*[115].

- **Regulation of *uhpT***
    - Transporter structure, expression, and transporter activity

Structurally, UhpT is a monomer with 12 α-transmembrane helices, matching the general structure of a large number of MFS transporters, such as GlpT. Arginine residues R46 and R275 are essential for the function of this transporter, as the others can be replaced by cysteine or lysine by targeted mutations. These residues (arginine) are highly conserved in this group of proteins[118].

Regulation of expression is mediated by a two-component system and a kinase[119]. Loss-of-function mutations in this transporter or in the genes of the regulatory system prevent bacterial growth in media whose only carbon source is G6P[120]. The two-component system is made up of UhpB and C, transmembrane proteins whose mission is to detect the presence of G6P in the periplasmic space (UhpC) and when this occurs, UhpB, which is phosphorylated, is able to yield its phosphate to UhpA, a kinase found in the cytosol[120,121]. The *uhpT* promoter has a binding site for phosphorylated UhpA, which acts as an inducer together with the cAMP-CRP complex [122].

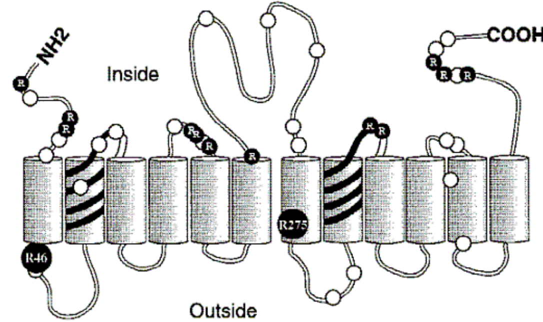


Figure 7. UhpT topology. The black circles represent arginines, highlighting the essential ones (R46 and R275). White circles represent lysines [118].

Different studies have found that phosphorylated UhpA acts as the essential element in the expression of *uhpT*, and that the cAMP-CRP complex acts as a cofactor of UhpA, increasing induction. Furthermore, there is another cofactor that can cause increased expression of this gene under anaerobic conditions, the FNR regulator also has a binding site to the *uhpT* promoter and, therefore, can also activate its expression. This regulator should not be the only factor that affects anaerobiosis, since even if *fnr* is mutated, *uhpT* still has a certain level of expression under this condition[123,124].

Reinforcing that the main inducer of *uhpT* is phosphorylated UhpA, a study shows that, in the absence of the two-component system, UhpBC, which detects the presence of G6P in the medium, UhpA can be activated independently in the presence of pyruvate. This is possible because UhpA can accept the phosphate group from acetyl phosphate, which is highly present during the growth on pyruvate via the Pta-AckA pathway[125]. However, the inorganic phosphate in the medium hinders transport[126,127].

Regardless of how it is regulated, the main function of this transporter is to introduce G6P, removing inorganic phosphate from the cytoplasm in an electrically neutral antiport reaction[128]. In addition, it can also incorporate other molecules: hexose phosphate and fosfomycin[107].

The integrity of this transporter is very important in the study of the susceptibility to fosfomycin. Early experiments with fosfomycin suggest that glucose-6-phosphate may be present in human tissues at sufficiently high concentrations to induce transcription of the *uhpT* transporter and thus increase the activity of the antimicrobial against organisms such as *E. coli* and *K. pneumoniae*[128]. Consequently, guidelines from committees such as CLSI and EUCAST state that the sensitivity of fosfomycin should be determined in the presence of 25mg/L glucose-6-phosphate to induce this transporter whenever it or its key regulators are present[129]

- **Regulation of *glpT***
  - Structure

Structurally, the GlpT transporter in *E. coli* is a protein with 12 α-transmembrane helices that form 2 domains connected by a loop where the opening allows the translocation of the substrate to the cytoplasm is located. The 12 helices, grouped from 2 domains of 6 helices (N- and C-terminal domains) present a pseudo-symmetry, in agreement with the weak homology presented by the two halves of the protein, indicating a gene duplication event in the evolution of the transporter. The two halves are connected by a long cytoplasmic loop. There are no salt bridges, and there are few hydrogen bonds between the domains, but there are many Van der Waals interactions in the periplasmic part of the protein. Importantly, there is a pore between the N- and C-terminal domains on the cytoplasmic side of the protein, which narrows towards the centre of the molecule. This represents the substrate translocation pore. The electrostatic potential of the GlpT surface shows an electroneutral pore surface, except at the closed end of the pore in the centre of the membrane, where it is positive. This area of strong positive charge is attributed to two conserved arginine residues, Arg45 of helix 1 and Arg269 of helix 7. These two arginines have also been shown to be essential for the activity of the *E. coli* UhpT protein. These conserved residues appear to form the substrate binding site for binding to the phosphate moiety of a substrate[130].

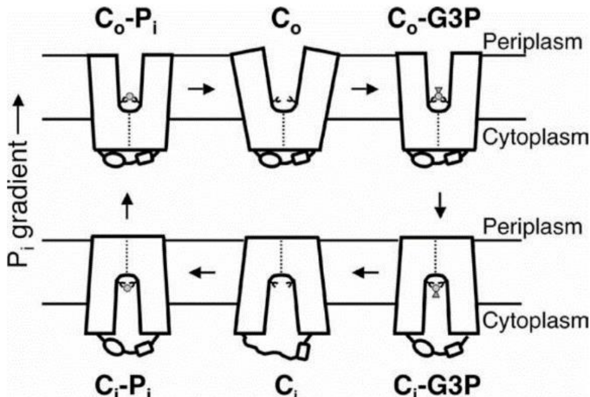


Figure 8. Mechanism of GlpT transport. The positions of the arginines of the binding site are indicated. The inorganic phosphate is shown as a small circle, and G3P as a triangle [130].

The structure of GlpT and biochemical data suggest that this transporter acts with a single binding site, alternating access with rocker-switch-like movement of the N- and C-terminal domains. Therefore, it requires two main conformations with the substrate binding site facing opposite sides of the bacterial membrane. In one study, it is proposed that inorganic phosphate binds to the arginines at the binding site, bringing the arginines and consequently the N- and C-terminal domains closer together, causing the pore to narrow. This binding would destabilise the interface between the two domains on the periplasmic side, allowing the conformational change. In the periplasmic space, the lower affinity of the protein for inorganic phosphate allows its substitution by G3P, while in the cytoplasm, phosphate substitutes G3P due to concentration[130,131].

- Transporter expression

The *glpT* gene is part of the *glp* regulon whose function is to regulate the entry and metabolism of glycerol and G3P. This regulon is made up of several operons (*glpABC*[132], *glpTQ*[133], *glpEGR*[134], *glpD*[135], and *glpFKX*[136] in *E. coli*), and its main regulator is the GlpR repressor, which binds to the promoter region of the operons and blocks their transcription[134].

GlpT transcription is induced by its own substrate (G3P), which binds to the GlpR repressor, releasing from the promoter region, thus a null mutation in the *glpR* gene activates a constutive *glpT* transcription. In studies by Larson *et al*., it was shown that G3P binds GlpR with an affinity K_d between 20-50 µM affinity, and that other glycerol-phosphate analogues could also bind to GlpR, while glycerol cannot do it. GlpR blocks the promoters of the different operons but binds strongly to those of *glpT* and *glpD* than to *glpFK*. The end products of lipid biosynthesis and glycolysis may provide the second site for *glpT* inhibition. On the other hand, inorganic phosphate in the medium hinders transport[126,127].

Other inducers of the *glpT* transporter would be the cAMP-CRP complex or the FNR regulator. As explained before, the cAMP-CRP complex has a binding site on the *glpT* promoter, binding when uptake of secondary carbon sources is needed and inducing its transcription[107]. In anaerobiosis, as is also the case for *uhpT*, the FNR regulator can also activate its expression, as it has an *fnr* site on the promoter. Furthermore, it seems that the binding affinity in the promoter is higher in the case of *glpT*, compared to *uhpT*[123,124]. This higher affinity may be due to the fact that G3P, a substrate transported by GlpT, can be used by the bacteria as an electron donor in anaerobic respiration. Under this condition, G3P is oxidized to dihydroxyacetone phosphate (DHAP) by glycerol-3-phosphate dehydrogenase, which in anaerobiosis situations is encoded by *glpABC*, and transfers electrons to terminal reductases. This would compensate for the loss of fitness that occurs in anaerobiosis[123].

- Glycerol-mediated induction

Glycerol is a triol that can be used as a carbon source. This molecule can enter the cytosol by passive diffusion, but also has a specific transporter, GlpF. Once inside the bacterium, glycerol is phosphorylated by the glycerol kinase (GlpK), producing intracellular G3P. This endogenous G3P can bind to GlpR, decreasing its affinity for promoters of the *glp* regulon and, in particular, *glpT*, and inducing expression[137,138].

This metabolic pathway in which glycerol is transformed into G3P within the bacterium occurs not only in *E. coli*, but also in other species such as *P. aeruginosa* or *P. putida*. What has also been observed is that the consumption of this carbon source is slow, resulting in a late and prolonged induction[139,140]. Thus, glycerol can act as an intracellular inducer of *glpT* expression.

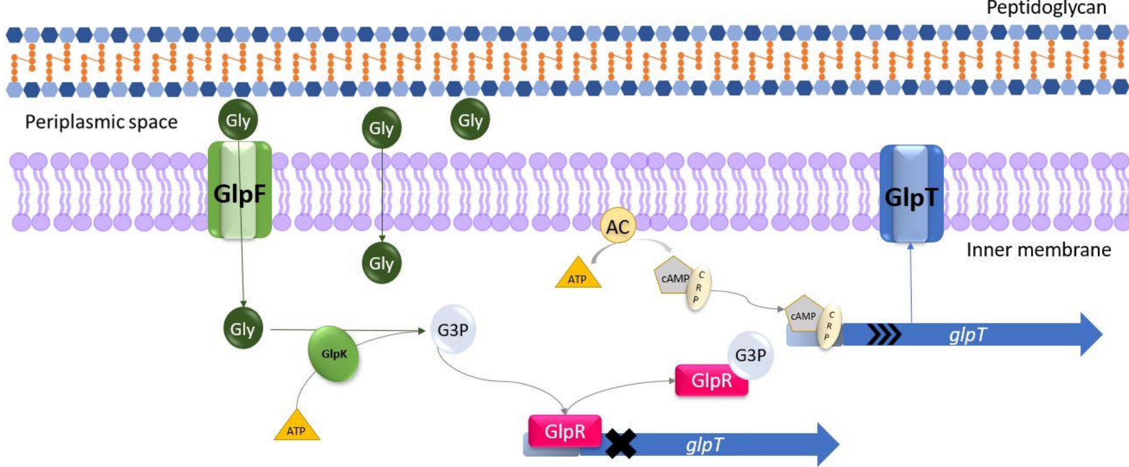


Figure 9. Representation of the transcriptional regulation system of the GlpT transporter by indirect induction of glycerol. Figure adapted and modified from Castañeda *et al*[107].

- Transporter activity

The main function of GlpT is the incorporation of G3P into the bacterium, removing inorganic phosphate. G3P is a molecule that can be used as a secondary carbon source and is essential in the biosynthesis of phospholipids. G3P is part of the backbone of all phospholipid molecules and the polar groups of phosphatidylglycerol and cardiolipin, which are indispensable in membrane formation[141]. The loss of function in this transporter means that bacteria suffering from it cannot grow on G3P as their sole carbon source[142]. In addition to G3P, GlpT allows the entry of other molecules such as phosphoenol-pyruvate or fosfomycin, although the affinity of the transporter for G3P is higher[143]. However, in contrast to activation of the UhpT transporter using G6P, the use of G3P also induces the expression of the GlpT transporter, but this molecule would compete with other substrates like fosfomycin for the occupancy of the transporter[143].

- **Phosphate homeostasis**

The control and metabolism of the inorganic phosphate plays an essential role for the bacterial physiology. As example, they are found at the hydrophilic ends of amphipathic lipids that form part of membranes; they are also present together with the ribose or deoxyribose sugars, forming a structural part of DNA and RNA. In addition, the cell's energy is based on the hydrolysis of phosphoanhydride bonds between the phosphates that form ATP, and the regulation of many biological systems are mediated by the phosphorylation or dephosphorylation of specific proteins [144]. All of this means that there are critical regulation systems to maintain optimal phosphate levels. In the case of *E. coli*, a range between 1 and 10mM has been observed with the participation of multiple transporter proteins with different expression patterns[145,146]. The main transporters that bring phosphate into the bacteria are PitA, PitB and PstSCAB systems; on the other hand, the transporters responsible for exporting inorganic phosphate are PitA, PitB, YjbB, GlpT, and UhpT[147–150]. This homeostasis is mainly controlled by the histidine kinase PhoR and the response regulator PhoB, which have been shown to regulate at least 31 genes, but this system is not directly involved in regulating *glpT* and *uhpT*[145]. Therefore, although the expression of the transporters is not regulated by the phosphate control system, the different concentrations to which the bacteria may be exposed can affect the activity of GlpT and UhpT, and can decrease transport under high phosphate conditions by going against the osmotic gradient.

4.5. Resistance mechanisms.

Fosfomycin resistance mechanisms may be intrinsically present in some bacterial species, or they may be acquired mechanisms. In both cases, fundamental resistance mechanisms affect the target (MurA) or the ability of the antimicrobial to reach the cytosol of the bacterium, where the target is located.

- **Intrinsic resistance**

There are bacteria that are intrinsically resistant to fosfomycin trough different mechanisms:

- Mutation in the *murA* gene

Some bacteria have mutations that produce an amino acid change at position 115, replacing the cysteine to which fosfomycin must bind with another amino acid. These mutations generate functional proteins, but lack the binding site for fosfomycin, leading to resistance to fosfomycin. This amino acid change has been described in bacteria such as *Vibrio fischeri*[151], *Mycobacterium tuberculosis*[152] and *Chlamydia spp*[153].

- Peptidoglycan recycling pathways

The other main mechanism of resistance to fosfomycin is intrinsic in the use of peptidoglycan recycling pathways. Some bacterial species can use alternative pathways of peptidoglycan synthesis, not requiring that it be created de novo by MurA. These alternative pathways have been described in *Acinetobacter baumannii*[154], *Pseudomonas aeruginosa* and *P. putida*[155,156].

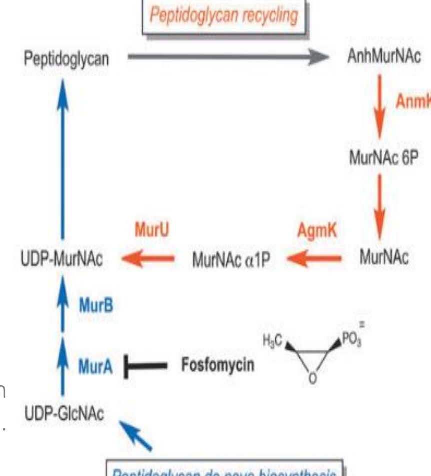


Figure 10: Simplified schematic of the peptidoglycan recycling pathway in *P. aeuroginosa*[155].

- Other intrinsic mechanisms

Listeria monocytogenes is resistant to fosfomycin in *in vitro* assays, as it is not able to incorporate this antimicrobial through its transporters. However, *in vivo*, this bacterial species is susceptible, because a virulence factor (hypoxanthine phosphoribosyltransferase) with G6P permease activity (like UhpT) is produced and can be used by fosfomycin to penetrate into the bacterium[157].

The constitutive decrease in membrane permeability of some species, such as *Acinetobacter baumannii*, is also considered an intrinsic mechanism of resistance to fosfomycin. This species has a chromosomal gene (*abrP*), which encodes a peptidase C13 family, whose deletion was able to increase cell membrane permeability and showing slower cell growth rate. This gene can reduce susceptibility to fosfomycin and other antimicrobials such as tigecycline, tetracycline, and chloramphenicol[158].

- **Acquired resistance**

  - Mutations in *murA*

Mutations in *murA* are exceptional in clinical isolates of *E. coli*. While no mutations have been found at position 115, the binding site of fosfomycin to MurA, some substitutions have been described in other positions (aspartic acid for asparagine at position 369 and leucine for isoleucine at position 370). Although not at the active site, these may interfere with the binding of fosfomycin to its target[159,160]. Also, an overexpression of *murA* gene, has been associated with fosfomycin resistance, but a bacterial fitness cost is observed under this phenotype [161].

Mutations in which a deletion at position 717 have also been found in *S. aureus*, decreasing the sensitivity to fosfomycin[162].

  - Decreased transport of fosfomycin

As discussed above, due to the location of the fosfomycin target (MurA), the antimicrobial must reach the bacterial cytoplasm [109]. Therefore, resistance to fosfomycin mainly depend on a decrease in

the uptake of fosfomycin. This decrease may occur through the acquisition of mutations that lead to loss of function in the transporters, or it may occur in other genes involved in their regulation[107]. As mentioned above, the expression of both transporters depends on induction by the presence of their own substrates (G6P and G3P), and on the binding of the cAMP-CRP complex[115]. The expression of both transporters is also increased by the presence of FNR, a global bacterial regulator that is activated under conditions of anaerobiosis[123,163].

It is important to note that not all the bacteria species have both transporters. As example, the Enterobacterales *E. coli* or *K. pneumoniae* present both permeases, however in the genus *Pseudomonas*, *P. aeruginosa* do not have the *uhpT* gen[164], while *P. putida* does not have the *glpT* gene[139].

The promoters of the *glpT* and *uhpT* genes present a binding site for the cAMP-CRP complex, which would act as an inducer of expression, so that a decrease in this complex would lead to a lower presence of the transporters and therefore a decrease in the entry of fosfomycin into bacteria causing fosfomycin resistance[115]. Loss-of-function mutations in the *cyaA* and *ptsI* genes, which are associated with decreased cAMP and thus decreased induction of fosfomycin transporters, increase resistance to fosfomycin and/or the frequency of emergence of fosfomycin-resistant mutants[165]. Because these mutations have a significant biological cost, they are rarely found in clinical isolates[166].

Also, loss-of-function mutations in *fnr*, one of the two main regulators in anaerobiosis, increase the resistance to fosfomycin 4-fold over the wild-type phenotype in anaerobiosis in *in vitro* experiments[123].

Mutations in *uhpT* or in the two-component system, *uhpBC, or* the transcriptional activator, *uhpA,* show a similar fosfomycin resistance phenotype[120]. On the other hand, while mutations in *glpT* do not increase the fosfomycin MIC value, the frequency fosfomycin resistant mutants increase. Furthermore, the combination of loss of function mutations in both transporters results in a greater increase in MIC than in the case of the *uhpT* mutation alone[165]. On the other hand, loss-of-function mutations in *glpR*, would lead to an increase in intracellular fosfomycin due to the resulting *glpT* overexpression[133].

- **Intrinsic and acquired resistance mechanisms**
  - Antibiotic-modifying enzymes

Increased resistance to fosfomycin may also be caused by the existence of enzymes that can modify the structure of the antimicrobial molecule, losing its antimicrobial properties. These enzymes

are present in the chromosome of certain microorganisms, in transposons, or plasmids. These can be divide into two groups: metalloenzymes and kinase-type enzymes[167].

The kinase-type enzymes are encoded by the *fomA* and *fomB* genes; their mechanism of action is phosphorylation of the fosfomycin molecule to inactivate it. FomA forms fosfomycin monophosphate and FomB uses this molecule as a substrate to obtain fosfomycin diphosphate. Both enzymes use ATP and Mg^{2+}. They are specific to bacteria that produce fosfomycin, such as *Streptomyces wedmorensis* and *Streptomyces fradiae*[168].

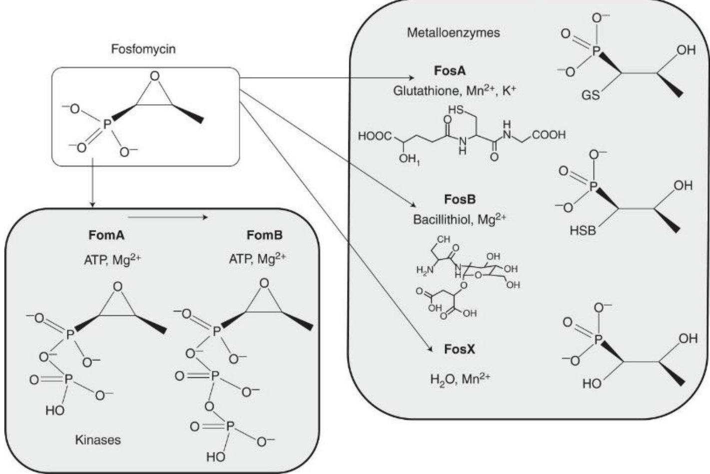


Figure 11. Main enzyme mechanisms of fosfomycin resistance[244].

On the other hand, the mechanism of action of metalloenzymes is to break the epoxide ring of the antimicrobial, requiring a metal as a cofactor. They are quite similar to each other, although they have some modifications that allow them to be classified[169].

- <u>*fosB*</u>:

The *fosB* gene codes for a metalloenzyme that catalyzes the addition of L-cysteine to fosfomycin, using Mg^{2+} as a cofactor, resulting in the fosfomycin molecule without bactericidal activity.

Regulation of the expression of this gene depends on the extracytoplasmic sigma factor, a factor involved in the induction of resistance mechanisms to other antimicrobials. The gene is present on the *Bacillus subtillis* chromosome and has been described in plasmids of *Staphylococcus spp* and *Enterococcus spp*[170].

- <u>*fosX*</u>:

The *fosX* gene codes for a metalloenzyme with hydrolase activity. Its mechanism of action is the addition of a water molecule at the C1 position of fosfomycin. The catalyst in this case would be glutamic acid and requires Mn^{2+} as a cofactor[171].

- *fosA*

The *fosA* gene codes for a glutathione-S-transferase, whose metal cofactor is manganese (Mn^{2+}). Its mechanism of action involves the nucleophilic addition of glutathione and K^+ to the epoxide ring of fosfomycin. Therefore, the presence of Mn^{2+} and K^+ is necessary for its action[172].

It was the first *fos*-type enzyme described and was found in a plasmid in *S. marcescens* in a plasmid. This gene has been described in the chromosome of bacteria such as *Klebsiella spp.*, *Enterobacter spp.*, *S. marcescens* and *P. aeruginosa*. Plasmid-encoded or plasmid-transformed chromosomal *fosA* has been shown to confer a high level of resistance to fosfomycin. Although it has significant variations in amino acid sequence, the residues forming part of the active site are highly conserved.

In Enterobacterales, there are different plasmid encoded *fosA* subtypes, of which *fosA3* is the most frequently detected in clinical isolates. It appears to be originated from the *Kluyvera georgiana* chromosome and is associated with plasmid transmission. Other important acquired subtypes are *fosA4*, *fosA5*, *fosA6*, *fosA8*, *fosA9* and *fosA10*[173]. The IS26 elements, which can mediate the integration of transposition units, are associated with *fosA3*, along with other resistance genes. This co-location can contribute as a reservoir of the spread of fosfomycin resistance and other resistance genes to clinically relevant pathogens[174].

Chromosomal *fosA* is part of *Klebsiella* spp. genome, being detectable in >99% of *K. pneumoniae* isolates. Furthermore, strains of *K. pneumoniae* and *K. variicola* have been found that, in addition to the chromosomal *fosA*, a plasmid-encoded *fosA* have been observed[172].

Different subtypes of *fosA*, both plasmid and chromosomal, confer different levels of resistance to fosfomycin, but all contribute to a lower fosfomycin susceptibility[175]. For example, most strains of *K. pneumoniae* strains have a MIC considered susceptible but borderline and depend on whether the use of the fosfomycin treatment is for urinary tract infection. Therefore, clinical use of fosfomycin may be limited against these species[172].

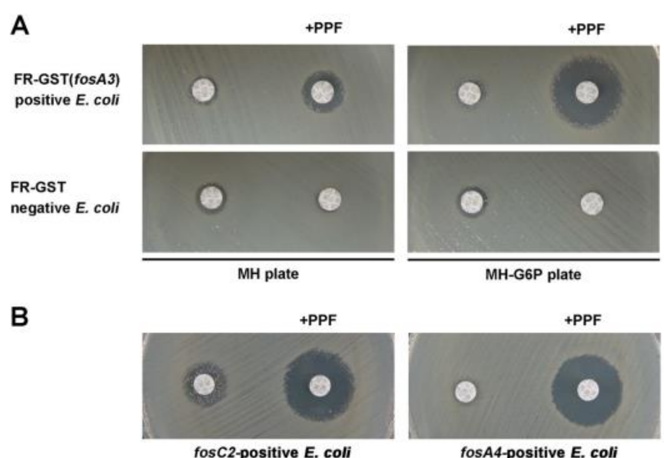
- <u>Sodium phosphonoformate</u>

The enzymatic activity of FosA is reduced by sodium phosphonoformate (PPF). This compound is a small molecule (126 Da), pyrophosphate analogue commonly used as an antiviral for the treatment of CMV retinitis in patients with acquired immunodeficiency syndrome (AIDS) and for treatment of acyclovir-resistant mucocutaneous HSV infections in immunocompromised patients. Its antiviral activity relies on the inhibition of the pyrophosphate binding site on virus-specific DNA polymerases at concentrations that do not affect cellular DNA polymerases[176]. PPF was first synthesized by Nylén in 1924 by alkaline hydrolysis of triethyl phosphonoformate although, the synthesis of trisodium phosphono [^{14}C] formate from [^{14}C] phosgene has been reported by Gawell in 1983. It has been found

Figure 12. Molecular structure of phosphonoformate

that phosphonoformate can be synthesized *in vivo* by *Streptomyces hygroscopicus* mutants[154] in a series of reactions starting with phosphoenolpyruvic acid.

Attempts have been made to test the ability to reduce the enzymatic activity of FosA with other phosphonates derivatives, but only PPF has shown remarkable activity. This is because it is the only one capable of forming a complex with the active site of the metalloenzyme, thus avoiding the addition of glutathione that inactivates fosfomycin[177].



This inhibitory capacity has been used to show the presence of plasmid encoded *fosA* in *E. coli.* This test consists in the addition of 1mg of PPF to the commercial (fosfomycin-G6P) antimicrobial susceptibility discs. If the isolate studied increases the inhibition zone compared to the same disc, but without the addition of PPF, it can be said that *fosA* is present[178].

Figure 13. Disk potentiation assay for detection of glutathione S-Transferase (FosA) production in *Escherichia coli* isolates[178].

4.6. Spectrum of action and epidemiology

Fosfomycin is considered as a broad-spectrum antimicrobial due to its activity against different bacterial species, including Gram-negative and Gram-positive bacteria. At the same time, it is a molecule whose efficacy is maintained against multidrug-resistant microorganisms[108].

- **Gram-positive spectrum of action**

Among Gram-positive bacteria, *S. aureus* shows fosfomycin susceptibility with variability between studies with respect to the percentages of susceptibility to fosfomycin of this microorganism, with values ranging from 33.2% to 100% of the isolates. Also, different species of *Enterococcus spp* also present a good response to this antimicrobial, but again the results vary according to the different studies[108]. In a study carried out on a total of 1,847 Gram-positive isolates from a focus other than the urinary tract, a general percentage of resistance to fosfomycin of 69.1% was observed. Analysing these data by the different bacterial species, 99.3% of *S. aureus* isolates, including MRSA, were susceptible to fosfomycin; however, lower susceptibility percentages were found in coagulase-negative staphylococci (77.5%), *Streptococcus pneumoniae* (61.9%), *Streptococcus pyogenes* (40.6%) and other Gram-positive (48.4%)[179]. Among Gram-positive microorganisms, the bacterial species *Staphylococcus saprophyticus*, *Staphylococcus capitis*, and *Corynebacterium spp*. are considered intrinsically resistant to fosfomycin[180].

- **Gram-negative spectrum of action**

In general, fosfomycin shows activity against Gram-negative microorganisms, mainly against enterobacteria of urinary origin. Studies show high sensitivity percentages for *E. coli* bacterial strains, ranging from 100% to 81%. However, lower percentages are usually observed for *K. pneumoniae*, with resistance values varying between 85% and 100% of the isolates, depending on the geographical areas[108].

Analyzing the fosfomycin susceptibility of other Enterobacterales, *Proteus spp* and *Enterobacter spp.* show higher percentages of resistance with respect to to *E. coli*, reaching values of 50% and 25%, respectively[108,181]. Contrary, *Citrobacter spp.* maintain high percentages of susceptibility, studies showing resistance percentages below 1%[182].

On the other hand, among the nonfermenting Gram-negative bacilli, *P. aeruginosa* and *A. baumannii* show decreased susceptibility to fosfomycin, with minimum inhibitory concentration (MIC) values between 16 mg/L and 64 mg/L[183]. Fosfomycin shows good activity against *Aeromonas hydrophila*, *Campylobacter jejuni*, and *Yersinia enterocolitica*, although against other species of the genera *Bordetella*, *Legionella*, *Pasteurella*, and *Vibrio*, its activity is moderate[184,185].

Furthermore, several studies have demonstrated fosfomycin activity against multidrug-resistant Gram-negative bacteria, especially in ESBL and carbapenemase-producing Enterobacterales; however, there are indications that resistance to this antibiotic is increasing in this type of microorganisms. In particular, a study conducted in Spain in more than 16,000 BLEE-producing *E. coli* strains between 2005 and 2011 showed a significant reduction in susceptibility to fosfomycin. The activity of fosfomycin during the last years of the study remained in fact above 80%, while resistance to other antimicrobials such as ciprofloxacin or cotrimoxazole was 78.2% and 62.3%, respectively, giving the impression that the phenomenon of co-resistance in BLEE-producing *Enterobacterales* is more related to other antibiotics than to fosfomycin[186]. Regarding *Enterobacterales* producing carbapenemase, most of the data come from studies conducted with KPC-type carbapenemase-producing isolates, with susceptibility percentages ranging from 39% to 100%. It should be noted that this antibiotic maintains its activity against Enterobacterales isolates that show resistance to other antimicrobials from the reserve group, such as colistin[103,108].

Regarding the activity of fosfomycin against nonfermenting Gram-negative bacteria considered as multidrug-resistant, such as *P. aeruginosa*, its susceptibility is very variable according to the different studies and according to geographical location[108]. Some *Acinetobacter* species, such as *A. baumannii*, *A. pittii*, and *A. nosocomialis* are considered intrinsically resistant to fosfomycin according to EUCAST

breakpoints, as well as other Gram-negative microorganisms such as *Burkholderia cepacia*, *Stenotrophomonas maltophilia*, or *Leclercia adecarboxylata*[180].

It has recently been shown that *fosA* is present in most of the chromosomes of species like *Klebsiella spp*, *Enterobacter spp*, *Serratia marcescens*, and *P. aeruginosa*, which would explain the decreased susceptibility of these microorganisms to fosfomycin. In contrast, they have not been found in the chromosome of *E. coli*, *A. baumannii* or *B. cepacia*, since the intrinsic resistance of these last two species is produced by other mechanisms[172].

- **Gram-negative epidemiology**

In Spain, resistance to fosfomycin in *E. coli* has varied over time. Two multicenter studies described an increase in resistance since 2003, both in ESBL-producing and non-producing isolates[187,188], but it is still very low and varies among the different autonomous communities. As an example, in the autonomous community of Aragón, the studies carried out showed resistance values to fosfomycin below 4% in isolates from community urinary tract infection between 2011 and 2013[189]. Similarly in Galicia, the percentages of fosfomycin susceptibility in *E. coli* isolates from urinary samples from different hospitals between 2011 and 2012, were higher than 95%, with the highest rates observed in patients older than 75 years of age of male sex, and the lowest in the paediatric population[190]. However, different studies have shown lower susceptibility values to fosfomycin in *K. pneumoniae*, especially in isolates that present other concomitant resistance mechanisms. A retrospective study conducted in Valladolid on the susceptibility profile of isolates of *E. coli* and *K. pneumoniae* from urine samples showed resistance values of 11.3% and 54%, respectively[191].

In Europe, a recent multicenter study showed resistance rates to fosfomycin in *E. coli* isolates of less than 1.2%[192]. Higher values have been reported in other European countries such as Germany (4.5%)[193], although good activity of this drug has been observed against other enterobacteria such as *Proteus mirabilis* or *Enterobacter spp.*, as well as against carbapenemase-producing Enterobacterales or ESBL-producing *E. coli*[193,194]. In a study carried out in France that analyzed 51,643 *E. coli* isolates of urinary origin, of which 3.3% were ESBL producers, and 3,495 isolates of *K. pneumoniae*, fosfomycin susceptibility higher than 95% was observed. In *E. coli* isolates from community and nosocomial urinary tract infections, resistance rates to fosfomycin were found to be 0-6% among those isolates producing ESBL, which, on the other hand, showed resistance rates of over 69% to other antibiotics, such as amoxicillin-clavulanic acid, quinolones or cotrimoxazole[195,196].

Globally, different studies have shown that overall resistance to fosfomycin in China is higher than in other parts of the world, both in isolates from human and animal samples, which could be

attributed to the presence and dissemination of the plasmid mediated *fosA3* gene, which is very prevalent in that geographical area[197,198].

4.7. Susceptibility studies

Susceptibility studies are performed to test the response of a microorganism against an antimicrobial, this type of *in vitro* assays is known as antibiogram and can be developed under. The antibiograms can evaluate the activity of one or more microorganisms or antimicrobials in a single assay. They are of great important because the result is used as a predictor of clinical efficacy. In 1961, the WHO published the methodology for performing sensitivity studies in a standardized manner, taking into account the different techniques[199].

Currently, the most important organizations responsible for evaluating and recommending antibiogram standards are the Clinical and Laboratory Standard Institute (CLSI) and the European Committee for Antimicrobial Susceptibility Testing (EUCAST). These organizations publish annual guidelines establishing the techniques and methodologies with which the most reliable results are obtained[200,201].

The most commonly used antibiogram techniques in microbiology laboratories are the following:

- **Agar dilution:**

In the agar dilution assay, an antimicrobial gradient is prepared in plates with agar culture medium. Decreasing antibiotic concentrations in Log2 dilutions are usually used. Each plate is inoculated by different microorganisms in spots; each spot must have an inoculum of 10^4 CFU. To automate this technique, a Steers replicator is usually used, an instrument that has wells where the microorganisms to be evaluated are placed, and tips that are immersed in these wells. These tips inoculate the plates with the different concentrations. Depending on the size of the tips, they inoculate approximately 2 µL or 0.15 µL, although regardless of the size used, there should be 10^4 colony-forming units (CFU) in each spot[202,203].

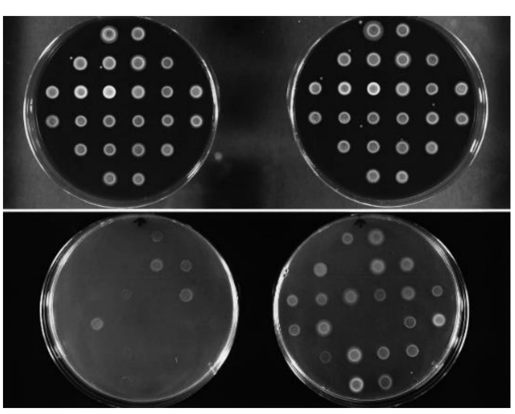


Figure 14. Agar plates on which the agar dilution technique has been performed[202].

The plates are incubated for 16-20 hours at 37°C. The media or incubation conditions in which this assay is performed depend on the antimicrobial and microorganisms evaluated. This technique is the reference standard for the evaluation of fosfomycin susceptibility for Enterobacterales using Mueller-Hinton II medium (with adjusted cations) together with 25 mg/L of G6P.

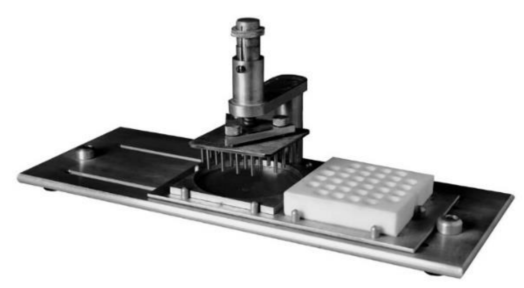


Figure 15. Steers replicator for inoculation in the agar dilution sensitivity testing technique[245].

This technique is interpreted by reading the inoculated spots at different antimicrobial concentrations and establishing the Minimum Inhibitory Concentration (MIC) value at the lowest concentration of fosfomycin that inhibits the bacterial growth discarding any single a colony or the presence of a faint bacterial growth halo[201].

- **Broth microdilution:**

The broth microdilution assay is the reference method for the determination of the MICs for most antibiotics. In this case, the antimicrobial concentration gradient performed in the liquid medium and each well contain an antimicrobial concentration. Similarly, as in the agar dilution assay, antimicrobial serial Log2 dilutions are evaluated. The bacterial inoculum should be according to standardized recommendations of 1-5 x 10^5 CFU/mL. After an incubation of 16-20 hours the plates are analysed and the MIC is established as the lowest antimicrobial concentration that inhibits the bacterial growth, i.e. no turbidity present in the well[203].

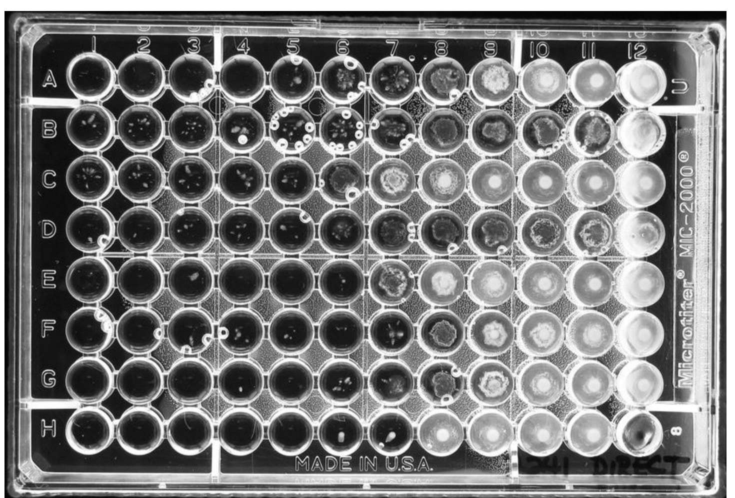


Figure 16. Microdilution plate showing inhibition of bacterial growth[246].

Most of the automated or semiautomated commercial methods are adaptations of this technique that simplify and speed up the preparation and allow testing a wide range of antimicrobials in a single assay.

- **Disc diffusion:**

In the disc diffusion technique, the antimicrobial is placed on a paper disc at a known amount. This paper disc is placed on a plate of solid medium previously inoculated with a bacterial concentration of 10^8 CFU/mL. The plate is incubated for 16 to 20 hours while the antibiotic diffuses through the agar, creating a concentration gradient forming a bacterial growth inhibition halo.

Figure 17. Antibiogram reading by measuring the diameter of the inhibition halo with the disc-diffusion technique[200].

Then the reading is made by measuring the diameter of the inhibition halos in millimeters. Multiple antimicrobials can be evaluated per plate; but only one microorganism can be present in each assay. Both, the CLSI and EUCAST committees, include this technique as reference method for the categorisation of the antimicrobial susceptibility.

This method offers a reliable and reproducible technique with an easy preparation and in reading. Also, the availability of a wide range of commercial discs with standardized amount antimicrobial permits a rapid implementation of this technique in the clinical microbiology routine. The main limitation of this technique is that a MIC value is not obtained as a result, however a corelation between millimeters of inhibition and MIC are performed in order to set the clinical breakpoints[200,201].

- **Gradient diffusion strips:**

In the gradient diffusion strip technique, the antimicrobial is placed on a plastic or paper strip in a concentration gradient of known and numbered concentration. The strip is placed on a plate of solid medium previously inoculated with a bacterial concentration of 10^8CFU/mL of the microorganism under study. The plate is incubated for 16 to 20 hours and the antibiotic diffuses through the agar, creating an exponential concentration gradient that forms an ellipse of inhibition of bacterial growth. The reading is made by taking as MIC value the interception of the bacterial growth with the strip.

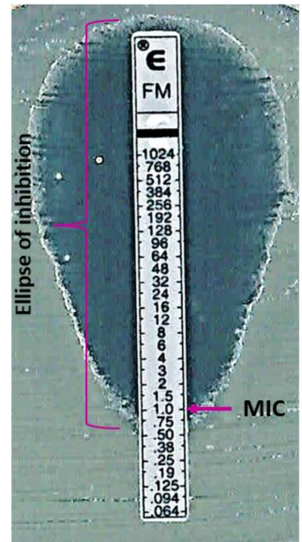


Figure 18. Fosfomycin gradient diffusion strip technique.

Figure 19. Gradient diffusion strip technique[245].

4.8. Clinical categories (Susceptibility breakpoints)

Susceptibility breakpoints are used to classify infection-causing microorganisms into clinical categories of susceptibility or resistance. Pathogens that are in the susceptible clinical category will have a higher probability of therapeutic success, while resistant pathogens will have a lower probability of therapeutic success. Depending on the type of antibiogram used, the susceptibility breakpoints points are expressed in concentration units in the case of antibiograms performed with a microdilution or agar dilution method that provide MIC values; or in distances in millimeters if the disc-diffusion technique is used[205].

It is important to understand that therapeutic success in a patient depends not only on the direct interaction of the microorganism with the antimicrobial, which is what is routinely evaluated in a microbiology laboratory, but also depends on immune factors, pharmacokinetics, pharmacodynamics, and other aspects. This makes necessary to periodically review and update the breakpoints that establish the clinical categories, modifying them if necessary, depending on new evidences on the factors mentioned above, that may impact in the clinical success of the antimicrobial treatment[205].

- Clinical breakpoints

The clinical breakpoints intended to mark the concentration of an antimicrobial that predicts the efficacy of a treatment using a standard dose during an infection caused by a certain microorganism[205,206]. There are no breakpoints for all possible combinations of antimicrobials and microorganisms; combinations of microorganisms and antimicrobials with intrinsic resistance are excluded, although other combinations are also excluded because there is insufficient evidence to establish them.

- In 2019 the definitions of clinical categories were modified. These categories are established based on the degree of exposure of the microorganism to the antimicrobial at the site of infection, which will depend on several factors such as mode of administration, dose, dosing interval, infusion time; as well as distribution, metabolism, and excretion of the drug. These categories are defined as[207]:
- *Susceptible to standard doses (S):* a microorganism is classified in this category when there is a high probability of therapeutic success using a standard antimicrobial agent dose regimen.
- *Susceptible to increased antimicrobial exposure (I):* a microorganism is classified in this category when there is a high probability of therapeutic success due to increased exposure to the antimicrobial agent due to adjustments in dosage or concentration of the agent in the focus of infection for physiological reasons.

- *Resistant (R):* a microorganism is classified as resistant when there is a high probability of therapeutic failure, even in a situation of increased exposure to the antimicrobial agent.

- **Epidemiologic Cut-Off: ECOFF**

The epidemiological cut-off values, or ECOFF, was established by EUCAST and is determined for a bacterial species against a specific antimicrobial. The ECOFF corresponds to the highest MIC value of isolates of the same species that do not present acquired resistance mechanisms against the antimicrobial and are therefore considered as wild-type strains[208]. They are determined by studying the MIC distribution curve of values evaluated in a large number of isolates for an antimicrobial by its reference method[209].

The clinical response to a treatment does not depend on the defined category by the epidemiological cut-off value, but they serve to monitor and detect the appearance of new resistance mechanisms. Mechanisms that may occur despite MIC values below the clinical breakpoints. ECOFFs, like clinical cut-off points, should be reviewed periodically. For example, in 2020, the ECOFF for fosfomycin against *E. coli* changed from 8 to 4 mg/L and in 2022 the ECOFF for *K. pneumoniae*, was set at 128 mg/L. However, it is not always possible to establish a cut-off value that separates the population without acquired resistance mechanisms from those with acquired resistance mechanisms[210].

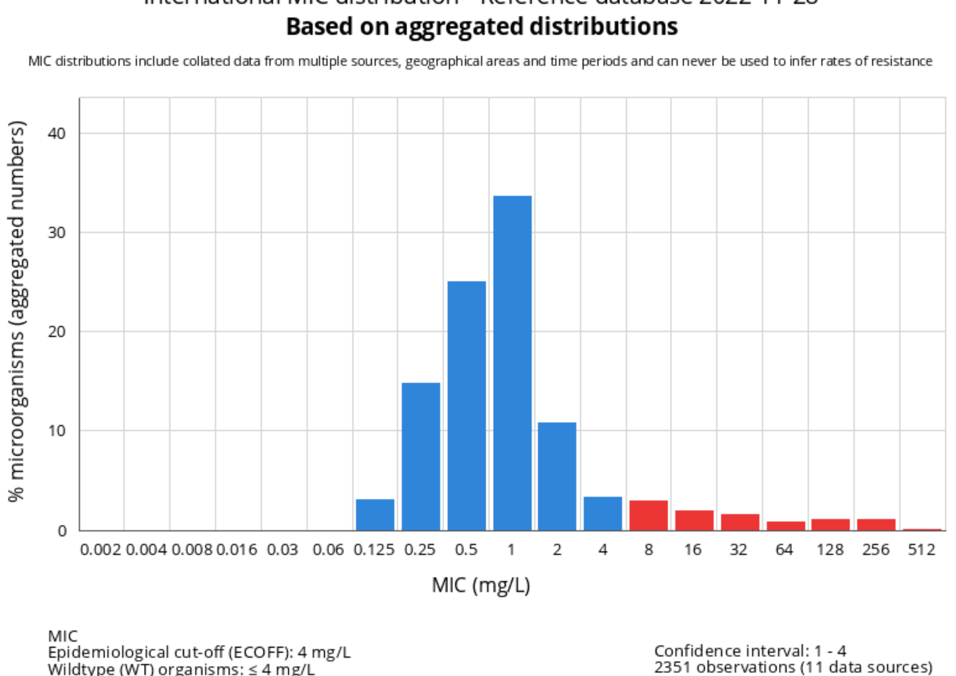
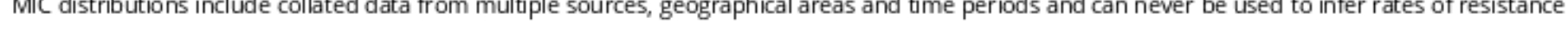


Figure 20. Distribution of fosfomycin MIC in 2351 *E. coli* isolates. ECOFF: 4 mg/L with confidence interval of 1-4[210].

Fosfomycin / Klebsiella pneumoniae
International MIC distribution - Reference database 2022-11-28
Based on aggregated distributions



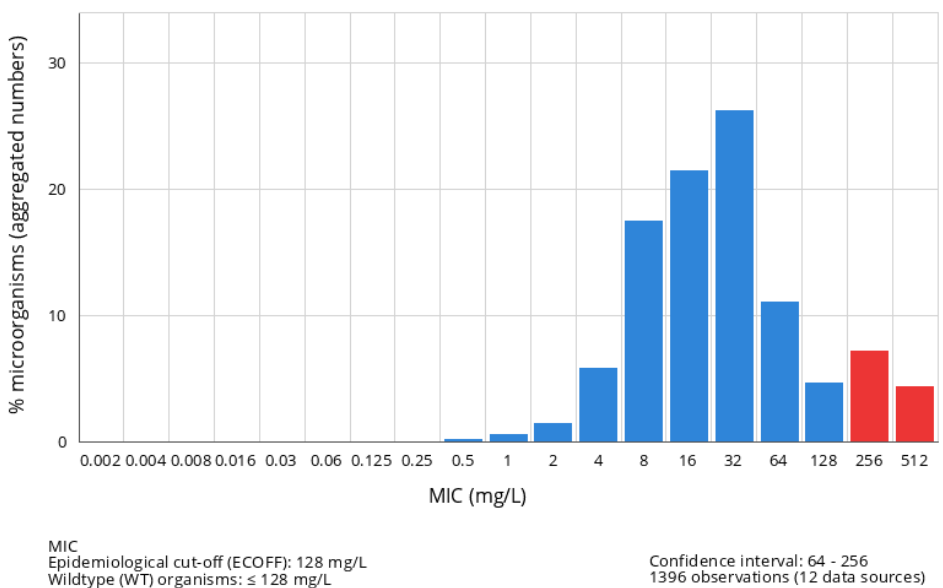


Figure 21. Distribution of fosfomycin MIC in 1396 *K. pneumoniae* isolates. ECOFF: 128 mg/L with confidence interval of 64-256[210].

In addition, it is important to keep in mind that the efficacy of an antimicrobial agent depends on the relationship between the MIC of the microorganism and the exposure of the microorganism to the agent in the patient. Thus, the clinical outcome depends on the triangular relationship between MIC, exposure, and efficacy. And in turn, the exposure of the microorganism to the antimicrobial agent in the patient depends on the dose and pharmacokinetic properties of the drug[211].

- **Fosfomycin susceptibility breakpoints**

According to CLSI and EUCAST, the agar dilution method supplemented with G6P at a concentration of 25 mg/L is the reference method for fosfomycin susceptibility studies and both recommend the addition of 50 µg of G6P for the sensitivity study with disc-diffusion of 200 µg of fosfomycin. However, they differ in the application and interpretation of the susceptibility breakpoints for this antimicrobial against Enterobacterales. EUCAST establishes that the study of susceptibility to fosfomycin by the disc diffusion method and/or techniques that provide MIC values is applicable to *E. coli*, while only the susceptibility breakpoints according to MIC can be applied to the rest of Enterobacterales. However, CLSI set the fosfomycin susceptibility breakpoints, both obtained by disc-diffusion and MIC, exclusively to *E. coli* of urinary origin[200,201].

The clinical susceptibility breakpoints for *Enterobacterales*, EUCAST establishes as susceptible those MIC values ≤8 mg/L and as resistant if >8 mg/L for an oral use of fosfomycin for the treatment of

uncomplicated urinary tract infection caused by *E. coli.* However, it establishes in susceptible category those MIC values ≤32 mg/L and as resistant if the concentration is >32 mg/L for the intravenous use of fosfomycin. On the other hand, CLSI set the categories of susceptible if the MIC is ≤64 mg/L, intermediate for a MIC of 128 mg/L and resistant when it is ≥256 mg/L. Similarly, the susceptibility breakpoints for disc-diffusion techniques differ between committees. According to the CLSI, those growth inhibition halos ≥16 mm are considered as susceptible, intermediate if the diameter is between 13-15 mm and resistant when the size is ≤12 mm. For EUCAST, those isolates with a growth inhibition halo ≥24 mm is considered as susceptible. EUCAST differentiates between the susceptibility breakpoints applied for the intravenous fosfomycin formulation from those applied to oral fosfomycin for the exclusive treatment in uncomplicated UTIs[200,201]. However, these breakpoints are under revision and will be soon harmonised[212].

Since 2017, in the breakpoint tables for interpretation of MICs and zone diameters of EUCAST published a section exclusively dedicated to the interpretation of inhibition zones in the fosfomycin disc-diffusion assays. These colonies that appear inside the inhibition zones can be considered as bacterial subpopulations with reduced susceptibility with respect to the rest of the bacterial population, showing a heteroresistance phenotype. However, the document does not mention the possible clinical implication or relevance of this phenomenon.

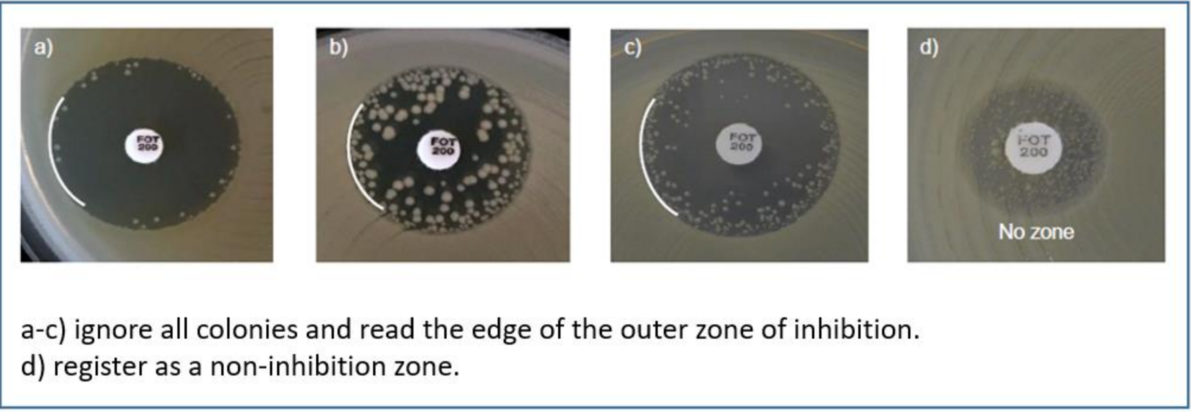


Figure 22. Examples to correct analysis and interpretation of inhibition zones in fosfomycin disc-diffusion assays against *E. coli*[200].

Contrary, CLSI has no interpretation for the inhibition zones in the presence of a heteroresistance phenotype for the study of fosfomycin susceptibility. CLSI simply states in general terms that in the presence of bacterial heteroresistance to an antimicrobial, the study with molecular techniques "may" be more appropriate than the phenotypic sensitivity study[213].

4.9. Subpopulations

The usual view of homogeneity in the metabolism of prokaryotic populations has been increasingly challenged in recent years, especially since the advent of single-cell technologies[214]. These methodologies have revealed the existence of a complete repertoire of responses to specific environmental conditions in individual microorganisms within the same population[215]. The diversification of metabolic pathways in single cells within otherwise clonal populations can be considered as a particular case of phenotypic variation, where different regulatory or epigenetic traits lead to the stochastic manifestation of alternative traits in isogenic individuals[216,217]. This allows phenomena such as persistence, i.e., the appearance of a fraction of living but nongrowing cells in a bacterial pool under a given circumstance, to occur, which is one of the most intriguing cases of phenotypic variation. Persistence ensures the survival of cells exposed to agents that act on growing bacteria, e.g., some antibiotics. Once the selective pressure is stopped, persistent bacteria can resume growth and completely rebuild the original population[218]. Also, the existence of subpopulations allows the phenomenon of heteroresistance[219]. Regardless of the mechanisms underlying these behaviours, the question that arises is whether these population differences are an adaptive trait or simply a chance occurrence that is beneficial for bacteria that are susceptible to the antimicrobial agents[139].

- **Heteroresistance**

The generalized definition of heteroresistance is the presence of a heterogeneous population of bacteria with one or more subpopulations with a decreased susceptibility to the antibiotic compared to the main population[219]. The genetic mechanisms leading to heteroresistance are multiple, and not all of them are well known. It should be noted that this definition encompasses a multitude of different underlying factors that bring different connotations to the heteroresistant bacterial population such as: the clonal origin of the bacterial population, the level of resistance of the subpopulations compared to that of the main population, the frequency of resistant subpopulations, and the stability of the heteroresistant phenotype (Figure 23)[220].

Depending on the clonal origin of the bacterial population, heteroresistance may be polyclonal or monoclonal. A bacterial clone is defined as those bacterial cells that arise from a single isolated CFU. Thus, polyclonal heteroresistance is defined when the heterogeneity in resistance of the bacterial population is due to the presence of different bacterial clones, genetically different, one susceptible, and the other resistant. In polyclonal heteroresistance, when the different bacterial clones are separated, isolated and purified in CFU, the heteroresistance phenomenon disappears. However, in monoclonal heteroresistance, the heterogeneity of resistance is generated from a single bacterial clone that differentiates into two different populations, one susceptible and the other resistant in the

absence of antibiotic pressure. The difference from the previous case is that the frequency with which resistant mutant populations spontaneously appear from the general susceptible population is very high. Therefore, a CFU contains millions of individual bacterial cells that has been formed from the division of a single bacterial cell, from a single clone, will in turn be composed of a population variety of resistant and susceptible cells. This heteroresistance phenotype will be maintained even when pure clones are analyzed as opposed to polyclonal[220].

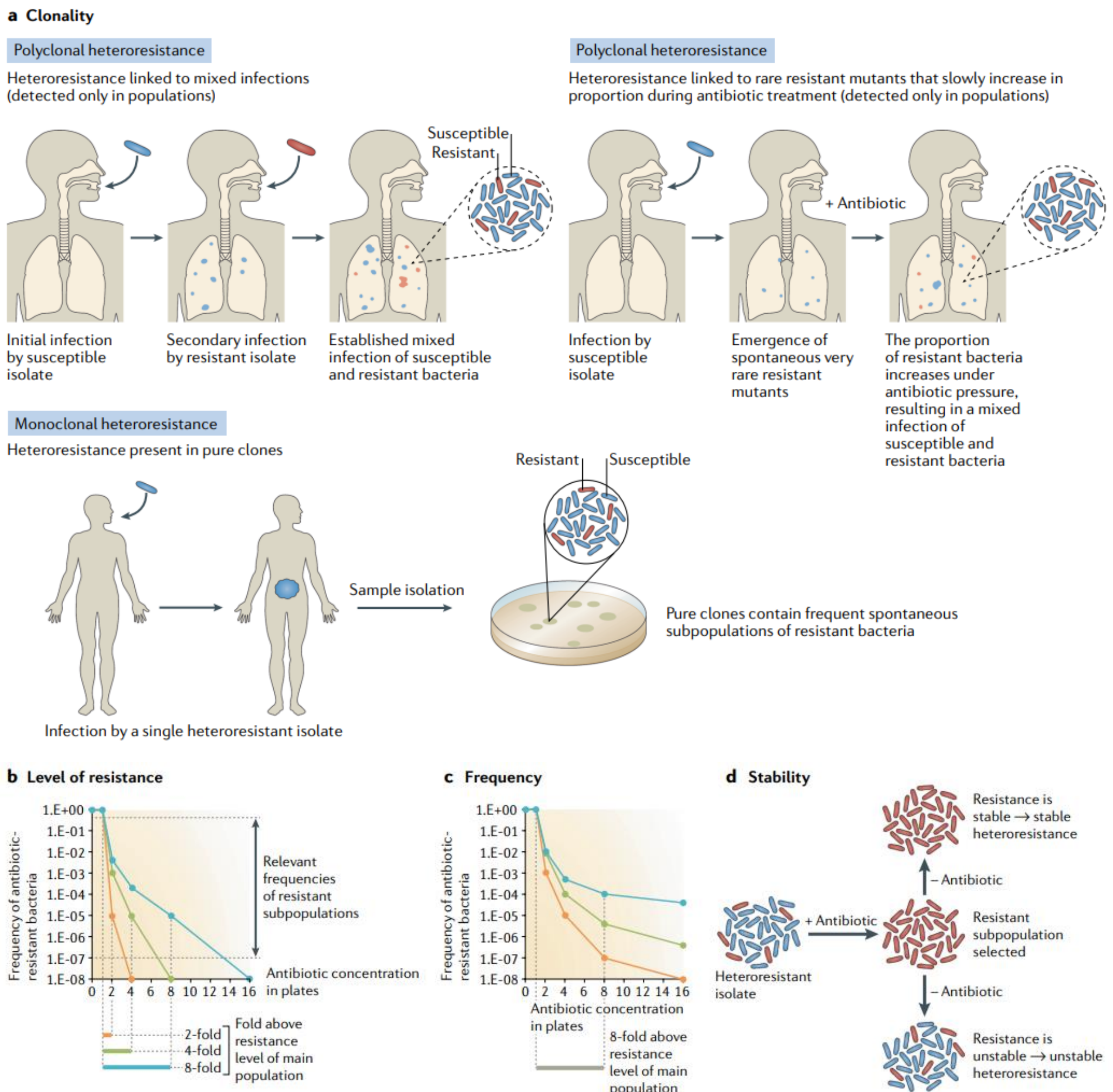


Figure 23. Factors to consider when defining heteroresistance[220].

Considering the level of resistance of the subpopulations with respect to the majority population, it can be said that most bacterial isolates can present subpopulations that grow at antibiotic concentrations close to their MIC. But there is an absence of criterium to define heteroresistance phenotype. Some studies define bacteria as heteroresistant when the MIC of the subpopulations are higher than 2 to 8 (base 2 serial dilutions) above the MIC of the majority population. Other definitions

include the presence of populations in which the resistant subpopulation exceeds the clinical susceptibility breakpoints established for the antibiotic[220].

The proportion or frequency of resistant subpopulations within the majority population is also a factor to be consider when defining an isolate as heteroresistant. This frequency indicates the number of resistant bacterial cells (or cells with decreased sensitivity) in the population. In the monoclonal heteroresistance, both the susceptibility and concentrations of the antibiotic used to study the resistant subpopulations and the minimum proportion established to consider these subpopulations as significant, will influence the classification of the isolate as heteroresistant. In the latest review on heteroresistance by Andersson *et al.*, they propose that the frequency of bacterial subpopulations should be reported at a concentration of antibiotic 8 times higher than the MIC of the main population[220].

The stability of the resistant subpopulations is another factor to be considered when defining heteroresistance, since this phenomenon can be stable or unstable and can affect the ability to be detect. Unstable heteroresistance is defined when resistant subpopulations can become susceptible again in the absence of antibiotic pressure after 50 generations in the absence of antibiotic[220].

The frequency of heteroresistance in clinical isolates depends on the definition used or the detection method; therefore, a great variability of heteroresistance rates is observed, ranging from 0% to 100% for the same combination of antibiotics and bacterial species, depending on the study. The frequency of heteroresistance to fosfomycin in clinical isolates has been studied in different bacterial species, and the same heterogeneous results have been observed[194,221,222,223,224].

IV. HYPOTHESES AND OBJECTIVES

The problem of antimicrobial resistance is limiting the treatability of infections caused by bacteria. Numerous strategies have been proposed to solve this problem, including the reintroduction of antimicrobials in disuse and the optimization of treatments. Therefore, more knowledge is needed on older antimicrobials, antibiotics to which bacteria remain sensitive, such as fosfomycin. These studies would allow an optimization of their use, in single treatment and in combination with other antimicrobials or adjuvants. Therefore, the hypotheses and objectives of this work are the following:

1. Hypothesis

I. Fosfomycin resistance in *Klebsiella pneumoniae* is explained by the presence of the fosfomycin inactivating enzyme chromosomal FosA together with inactivation of the fosfomycin transporter GlpT and UhpT.

II. Fosfomycin susceptibility in *K. pneumoniae* can be restored using sodium phosphonoformate as FosA inhibitor.

III. Fosfomycin activity can be enhanced activating *glpT* transcription increasing glycerol-3-phosphate intracellular concentrations with glycerol in *E. coli*.

IV. Inorganic phosphate extracellular concentrations modify *E. coli* fosfomycin susceptibility at human physiological concentrations.

2. Objectives

I. To study the interaction between the main determinants of fosfomycin resistance in *K. pneumoniae*.

II. To evaluate the activity of sodium phosphonoformate (PPF) as a FosA inhibitor in *K. pneumoniae*.

III. To determine the effect of glycerol as an inducer of the *glpT* transporter at therapeutic concentrations and to understand the effect of glycerol on the activity of fosfomycin at therapeutic concentrations against *E. coli*.

IV. To study whether the physiological concentrations of phosphate can affect the transport of fosfomycin in *E. coli*.

V. PUBLICATIONS

1. Interplay among Different Fosfomycin Resistance Mechanisms in *Klebsiella pneumoniae*.

Infections with extended-spectrum beta-lactamase (ESBL)-producing *Klebsiella pneumoniae* are of particular concern. Fosfomycin is an antimicrobial currently approved in several countries for the treatment of uncomplicated urinary tract infections caused by *Enterobacteriaceae*, with activity against multidrug-resistant strains of *K. pneumoniae*. However, the study of fosfomycin resistance determinants in *K. pneumoniae* has not been sufficiently studied.

Therefore, the first article of the work aimed to determine the in vitro interaction between the main determinants of fosfomycin resistance and fosfomycin resistance in *K. pneumoniae*. In addition, we also evaluated the possibility of using PPF as an adjuvant of fosfomycin in the treatment against *K. pneumoniae*.

The results obtained in this work are the following:

Fosfomycin resistance-related gene sequences.

The results for fosfomycin resistance-related proteins (GlpT, UhpT, FosA, CyaA, Crp, PtsI, MurA, UhpA, UhpB, and UhpC) after de novo sequencing of the 12 *K. pneumoniae* isolates are shown in Table 1 (Art.1). No amino acid changes in CyaA, Crp, MurA, or UhpA were found in these clinical isolates compared with K. pneumoniae ATCC 700721. Apart from polymorphisms with activity whose significance is unknown, GlpT insertions were found in Kp58, Kp88 and Kp108, and deletions were detected in Kp86. The clinical isolate Kp88 also showed a truncated UhpT protein due to a premature stop codon.

Fosfomycin sensitivity.

The fosfomycin MICs for the isogenic collection and clinical isolates are shown in Table 1 (Art.1). The modal MIC of the clinical isolates was 32 mg/L, and the MICs of fosfomycin ranged from 16 to 1,024 mg/L. For isogenic mutants, similar results were obtained for *K. pneumoniae* ATCC 700721, Kp12 and Kp142. The DglpT mutant showed no increase in MIC relative to that of the wild-type (WT) strain (32 mg/L), whereas inactivation of uhpT led to a 32-fold increase. Deletion of fosA led to a change in MIC 32-fold decrease relative to wild-type (1 mg/L).

Addition of 0.623mM PPF reduced the MIC values of fosfomycin against all clinical isolates by 2- to 8-fold compared to no PPF. Δ*uhpT* and Δ*fosA* strains from the isogenic collection showed no decrease in fosfomycin MIC with the addition of PPF. Furthermore, the MICs of fosfomycin against Kp12 and Kp142 Δ*fosA* strains were 4-fold and 8-fold higher, respectively.

Synergy assays.

The combination of fosfomycin and PPF showed synergistic activity against *K. pneumoniae* ATCC 700721, Kp12 and Kp142 strains (Fig. 1, Art.1). According to the synergy score (ZIP), the mean (and

maximum) bacterial response ratios attributable to the drug interaction were 15.46 (42.96), 26.88 (65.15) and 30.7 (65.77) for *K. pneumoniae* ATCC 700721, Kp12 and Kp142, respectively. This maximum synergistic zone occurred at fosfomycin ranges of 2 to 32 mg/L, and at PPF ranges of 0.3 to 2.5mM.

Mutant frequencies.

The results of mutant frequency estimates are shown in Fig. 2 (Art.1). The lowest frequencies of fosfomycin-resistant mutants were found among the Δ*fosA* mutants with a decrease in frequency from 1.69×10^{-1} to 1.60×10^{-5} at 64 mg/L and from 5.38×10^{-3} to 2.13×10^{-5} at 512 mg/L relative to their wild-type strains. In terms of fosfomycin mutant selection, little or no differences were observed between Δ*glpT* and wild-type strains. The Δ*uhpT* mutation showed a frequency close to 1, confirming the existence of a uniform population with an MIC above the selection conditions of fosfomycin. The addition of PPF had no effect on mutant frequencies (Fig. S1, Art.1).

Bacterial growth monitoring.

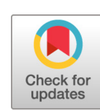
Figure 3 and Fig. S2 (Art.1) show the results of the 24-h growth monitoring assays. With respect to the isogenic collection of mutants (Fig. 3, Art.1), the Δ*fosA* strains showed the highest susceptibility after exposure to fosfomycin, with no growth at concentrations of 32, 8 and 4 against K. pneumoniae ATCC 700721, Kp12 and Kp142, respectively. High viability of the Δ*glpT* and Δ*uhpT* mutants was observed across the range of fosfomycin concentrations tested. Finally, the addition of PPF did not reduce the viability of any mutant except the Kp12 Δ*glpT* mutant. With respect to clinical isolates, in susceptible strains without known fosfomycin resistance-related mutations, viability was not observed at 256, 256 and 128 mg/L, respectively (Fig. S2, Art.1). The addition of PPF showed an increase in fosfomycin activity, reducing by 3 log2 dilutions the concentration of fosfomycin capable of killing viable bacteria. Although the isolates were susceptible but had mutations in GlpT, none of the tested fosfomycin concentrations were able to eradicate bacterial growth. Similar results were obtained with the highly resistant strains. No differences in viability were observed with the addition of PPF against isolates with mutations or resistant.

Time-kill assays.

K. pneumoniae ATCC 700721, Kp12, Kp142, and their derivatives at two concentrations of fosfomycin (64 and 307 mg/L), with and without 0.623mM PPF. The results of the assays are shown in Table 2 and Figs. S3, S4 and S5 (Art.1).

Heterogeneous bactericidal effect of fosfomycin was observed at both concentrations studied, with a bactericidal effect or decrease in bacterial load during the first hours of the study, in all strains except for the *uhpT* gene mutant. In all cases (except Kp142 at 307mg/L and mutants for the fosA gene) there is regrowth at the end of the assay. The bactericidal effect was not increased by the addition of PPF.

MECHANISMS OF RESISTANCE



Interplay among Different Fosfomycin Resistance Mechanisms in *Klebsiella pneumoniae*

M. Ortiz-Padilla,[a,b,c,d] I. Portillo-Calderón,[a,c,d] B. de Gregorio-Iaria,[a] J. Blázquez,[d,e] J. Rodríguez-Baño,[a,c,d,f] A. Pascual,[a,b,c,d] J. M. Rodríguez-Martínez,[b,c,d] F. Docobo-Pérez[b,c,d]

[a]Unidad de Gestión Clínica de Enfermedades Infecciosas, Microbiología y Medicina Preventiva, Hospital Universitario Virgen Macarena, Seville, Spain
[b]Departamento de Microbiología, Universidad de Sevilla, Seville, Spain
[c]Instituto de Biomedicina de Sevilla IBIS, HUVM/HUVR/CSIC/Universidad de Sevilla, Seville, Spain
[d]Red Española de Investigación en Patología Infecciosa (REIPI RD16/0016), Instituto de Salud Carlos III, Madrid, Spain
[e]Centro Nacional de Biotecnología, Madrid, Spain
[f]Departamento de Medicina, Universidad de Sevilla, Seville, Spain

J. M. Rodríguez-Martínez and F. Docobo-Pérez contributed equally as senior coauthors.

ABSTRACT The objectives of this study were to characterize the role of the *uhpT*, *glpT*, and *fosA* genes in fosfomycin resistance in *Klebsiella pneumoniae* and evaluate the use of sodium phosphonoformate (PPF) in combination with fosfomycin. Seven clinical isolates of *K. pneumoniae* and the reference strain (ATCC 700721) were used, and their genomes were sequenced. Δ*uhpT,* Δ*glpT,* and Δ*fosA* mutants were constructed from two isolates and *K. pneumoniae* ATCC 700721. Fosfomycin susceptibility testing was done by the gradient strip method. Synergy between fosfomycin and PPF was studied by checkerboard assay and analyzed using SynergyFinder. Spontaneous fosfomycin mutant frequencies at 64 and 512 mg/liter, *in vitro* activity using growth curves with fosfomycin gradient concentrations (0 to 256mg/liter), and time-kill assays at 64 and 307 mg/liter were evaluated with and without PPF (0.623 mM). The MICs of fosfomycin against the clinical isolates ranged from 16 to ≥1,024 mg/liter. The addition of 0.623 mM PPF reduced fosfomycin MIC between 2- and 8-fold. Deletion of *fosA* led to a 32-fold decrease. Synergistic activities were observed with the combination of fosfomycin and PPF (most synergistic area at 0.623 mM). The lowest fosfomycin-resistant mutant frequencies were found in Δ*fosA* mutants, with decreases in frequency from 1.69×10^{-1} to 1.60×10^{-5} for 64 mg/liter of fosfomycin. In the final growth monitoring and time-kill assays, fosfomycin showed a bactericidal effect only with the deletion of *fosA* and not with the addition of PPF. We conclude that *fosA* gene inactivation leads to a decrease in fosfomycin resistance in *K. pneumoniae*. The pharmacological approach using PPF did not achieve enough activity, and the effect decreased with the presence of fosfomycin-resistant mutations.

KEYWORDS *Klebsiella pneumoniae*, antimicrobial resistance, fosfomycin

The reported worldwide increase in antibiotic resistance, together with the shortage of new active drugs, has made it necessary to reuse old antimicrobial agents as an alternative strategy (1, 2). As a result, interest in fosfomycin has increased, with the aim of obtaining a better understanding of its pharmacokinetic (PK) and pharmacodynamic (PD) properties and its effectiveness in difficult-to-treat infections caused by multidrug-resistant *Enterobacteriaceae* (3, 4).

Extended-spectrum beta-lactamase (ESBL)-producing *Klebsiella pneumoniae* infections are particularly worrisome, as the incidence of such infections has increased dramatically, with limited therapeutic options for patients (5). Fosfomycin is an antimicrobial currently approved in several countries for treating uncomplicated urinary tract infections caused by *Enterobacterales*, with activity against multidrug-resistant *K. pneumoniae* strains (6).

Citation Ortiz-Padilla M, Portillo-Calderón I, de Gregorio-Iaria B, Blázquez J, Rodríguez-Baño J, Pascual A, Rodríguez-Martínez JM, Docobo-Pérez F. 2021. Interplay among different fosfomycin resistance mechanisms in *Klebsiella pneumoniae*. Antimicrob Agents Chemother 65:e01911-20. https://doi.org/10.1128/AAC.01911-20.

Copyright © 2021 American Society for Microbiology. All Rights Reserved.

Address correspondence to F. Docobo-Pérez, fdocobop@yahoo.es.

Received 4 September 2020
Returned for modification 19 October 2020
Accepted 18 December 2020
Accepted manuscript posted online 23 December 2020
Published 17 February 2021

Downloaded from http://aac.asm.org/ on March 5, 2021 at USE/BCTA.GEN UNIVERSITARIA

TABLE 1 Fosfomycin MIC by gradient strip assay, with and without the addition of 0.623 mM PPF and amino acid modifications in fosfomycin resistance-related proteins relative to reference strain *K. pneumoniae* ATCC 700721[a]

	MIC (mg/liter)		Mutation					
Strain	**Without PPF**	**With PPF**	***glpT***	***uhpT***	***fosA***	***ptsI***	***uhpB***	***uhpC***
ATCC 700721	32	8						
ATCC 700721 Δ*fosA*	1	2			Δ*fosA*			
ATCC 700721 Δ*glpT*	32	8	Δ*glpT*					
ATCC 700721 Δ*uhpT*	≥1,024	≥1,024		Δ*uhpT*				
Kp12	32	4		**A462T**		**N174K**		**L337M**
Kp12 Δ*fosA*	1	4		A462T	Δ*fosA*	N174K		L337M
Kp12 Δ*glpT*	32	8	Δ*glpT*	A462T		N174K		L337M
Kp12 Δ*uhpT*	≥1,024	≥1,024		Δ*uhpT*		N174K		L337M
Kp142	32	8		**A462T**		**N174K**		**L337M**
Kp142 Δ*fosA*	1	8		A462T	Δ*fosA*	N174K		L337M
Kp142 Δ*glpT*	32	16	Δ*glpT*	A462T		N174K		L337M
Kp142 Δ*uhpT*	≥1,024	≥1,024		Δ*uhpT*		N174K		L337M
Kp28	32	4		A462T		N174K		L337M
Kp58	32	16	A261_N262*ins*IA	A462T		N174K		L337M
Kp86	≥1,024	256	I260_A261*del*	A462T	D138E	N174K		
Kp88	16	2	A261_N262*ins*IA	A462T		N174K		L337M
Kp108	≥1,024	128	Y258_I259*ins*IA	A462T		N174K	T140A	L337M

[a]Data for wild-type ATCC 700721, Kp12, and Kp142 are in bold. *ins* and *del*, insertion and deletion, respectively.

Fosfomycin disrupts the first step in peptidoglycan biosynthesis by inhibiting the UDP-*N*-acetylglucosamine-3-enolpyruviltransferase (MurA) enzyme leading to cell death. To carry out its activity, fosfomycin enters the bacteria via the membrane transporters UhpT (a hexose phosphate transporter) and GlpT (glycerol-3-phosphate transporter) (7). The loss of function of these transporters or genes involved in their regulation is the most common mechanism of resistance in *Escherichia coli* clinical isolates (8). Together with these resistance mechanisms, chromosomal or plasmid-borne fosfomycin-inactivating enzymes are present in many Gram-negative bacteria (9). The *fosA* gene encodes a metallo-glutathione *S*-transferase, widely distributed in the genomes of Gram-negative bacteria, mostly those belonging to the family *Enterobacterales*, such as *K. pneumoniae*. Plasmid-encoded or chromosomal *fosA* transformed into high-copy-number plasmids has been shown to confer high-level fosfomycin resistance (9, 10). The enzyme activity of this protein is reduced by sodium phosphonoformate (PPF), a pyrophosphate analogue used for the treatment of cytomegalovirus and herpes simplex virus due to its capacity to inhibit viral DNA polymerases (11). It has been used to detect plasmid-borne *fosA* (11), but its antimicrobial activity in combination with fosfomycin against *fosA*-bearing strains has also been explored (12).

Nevertheless, the contribution of this fosfomycin resistance determinant alone and in combination with mutations affecting fosfomycin resistance-related genes remains unclear.

The present study aimed to determine the *in vitro* interplay between the main fosfomycin resistance determinants and fosfomycin resistance in *K. pneumoniae*.

(This study was presented in part at ECCMID 2019; Amsterdam, Netherlands [poster presentation P1903].)

RESULTS

Sequences of fosfomycin resistance-related genes. The results for the fosfomycin resistance-related proteins (GlpT, UhpT, FosA, CyaA, Crp, PtsI, MurA, UhpA, UhpB, and UhpC) after *de novo* sequencing of the 12 *K. pneumoniae* isolates are shown in Table 1. No amino acid changes were found in CyaA, Crp, MurA, or UhpA in these clinical strains compared with *K. pneumoniae* ATCC 700721. Apart from polymorphisms with activity whose significance is unknown, insertions in GlpT were found in Kp58 (A261_N262*ins*IA), Kp88 (A261_N262*ins*IA), and Kp108 (Y258_I259*ins*IA) and

Downloaded from http://aac.asm.org/ on March 5, 2021 at USE/BCTA.GEN UNIVERSITARIA

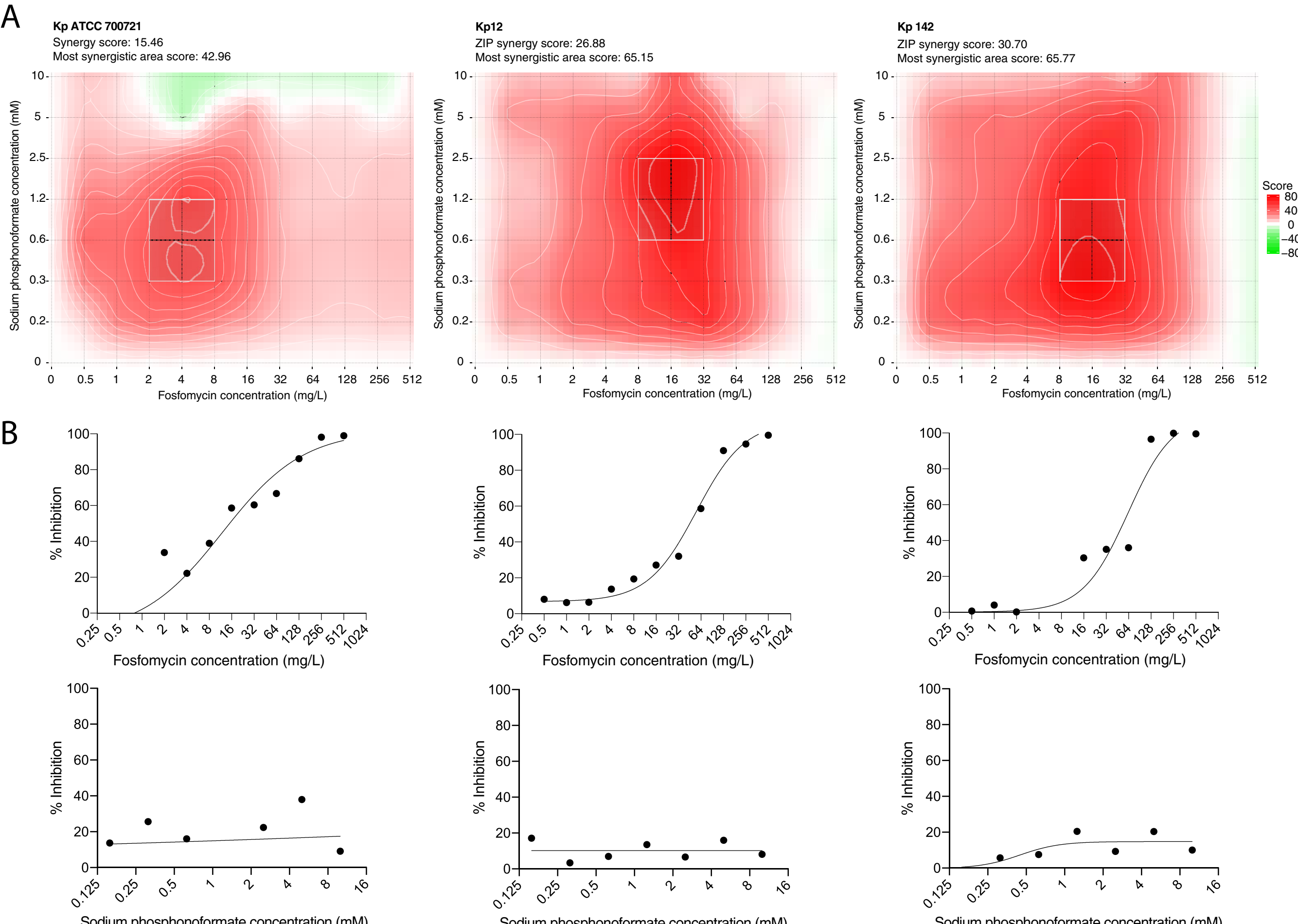


FIG 1 (A) Synergistic activity of fosfomycin in combination with PPF against *K. pneumoniae* ATCC 700721, Kp12, and Kp142 strains. Red and green areas represent synergy (synergy score greater than +10) and antagonism (less than −10), respectively. White rectangles show the maximum synergy area. (B) Fosfomycin and PPF dose-response curves.

deletions were detected in Kp86 (I260_A261*del*). The clinical Kp88 isolate also showed a truncated UhpT protein due to a premature stop codon.

Fosfomycin susceptibility. The fosfomycin MICs for the isogenic collection and clinical isolates are shown in Table 1. The modal MIC of the clinical isolates was 32 mg/liter, and the fosfomycin MICs ranged from 16 to ≥1,024 mg/liter. For the isogenic mutants, similar results were obtained for *K. pneumoniae* ATCC 700721, Kp12, and Kp142. The deletion of *glpT* showed no increase in MIC relative to that of the wild-type (WT) strain (32 mg/liter), while inactivation of *uhpT* (MIC of ≥1,024 mg/liter) led to a ≥32-fold increase. Deletion of *fosA* caused a MIC change to 1 mg/liter, a 32-fold decrease with respect to that of the wild type.

The addition of 0.623 mM PPF reduced the MIC values of fosfomycin against all clinical isolates between 2- and 8-fold compared with the absence of PPF. The Δ*uhpT* and Δ*fosA* strains in the isogenic collection showed no decrease in fosfomycin MIC with the addition of PPF. Also, the fosfomycin MICs against Kp12 and Kp142 Δ*fosA* strains were 4- and 8-fold higher, respectively. Finally, Δ*glpT* mutants of *K. pneumoniae* ATCC 700721 and Kp12 showed a 4-fold reduction in the fosfomycin MIC.

Synergy assays. The combination of fosfomycin and PPF showed synergistic activity against *K. pneumoniae* ATCC 700721, Kp12, and Kp142 strains (Fig. 1). According to the synergy score, the average (and maximum) proportions of bacterial response attributable to the drug interaction were 15.46 (42.96), 26.88 (65.15), and 30.7 (65.77) for *K. pneumoniae* ATCC 700721, Kp12, and Kp142, respectively. This maximum synergistic

Downloaded from http://aac.asm.org/ on March 5, 2021 at USE/BCTA.GEN UNIVERSITARIA

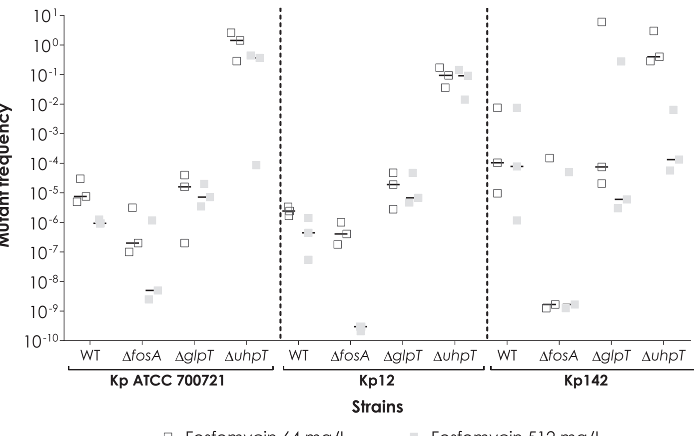


FIG 2 Fosfomycin-resistant mutant frequencies of *K. pneumoniae* ATCC 700721, Kp12, and Kp142 wild-type and isogenic mutant strains. Empty and full boxes show individual results, and black lines represent the median values of the three replicates.

area occurred in fosfomycin ranges of 2 to 8 mg/liter for *K. pneumoniae* ATCC 700721 and 8 to 32 mg/liter for Kp12 and Kp142 and in PPF ranges of 0.3 to 1.2 mM for *K. pneumoniae* ATCC 700721 and Kp142 and 0.6 to 2.5 mM for Kp12.

Mutant frequencies. The results of the mutant frequency estimates are shown in Fig. 2. The lowest fosfomycin resistance mutant frequencies were found among Δ*fosA* mutants, with a decrease in frequency from 1.69×10^{-1} to 1.60×10^{-5} at 64 mg/liter and from 5.38×10^{-3} to 2.13×10^{-5} at 512 mg/liter relative to their wild-type strains. In terms of fosfomycin selection of mutants, small or no differences were observed between Δ*glpT* and wild-type strains at 64 or 512 mg/liter. The Δ*uhpT* mutation showed a frequency of close to 1, confirming a uniform population with a MIC above the selecting conditions, i.e., 64 or 512 mg/liter of fosfomycin. Addition of PPF showed no effect on mutant frequencies (see Fig. S1 in the supplemental material).

Bacterial growth monitoring. Figure 3 and Fig. S2 show the results for 24-h-growth monitoring assays, expressed as percentage of bacterial viability at each fosfomycin concentration used. The experiment was carried out with the isogenic collection and clinical isolates.

With respect to the isogenic collection of mutants (Fig. 3), Δ*fosA* strains showed the highest susceptibility after fosfomycin exposure, with absence of growth at concentrations of 32, 8, and 4 against *K. pneumoniae* ATCC 700721, Kp12, and Kp142, respectively. High viability of Δ*glpT* and Δ*uhpT* mutants was observed across the range of fosfomycin concentrations tested. Finally, the addition of PPF did not reduce the viability of any mutant, except for the Kp12 Δ*glpT* mutant.

With respect to the wild-type clinical isolates, in the susceptible strains without known fosfomycin resistance-related mutations (*K. pneumoniae* ATCC 700721, Kp12, Kp28, and Kp142), no viability was observed at 256, >256, 256, and 128 mg/liter, respectively (Fig. S2). The addition of PPF showed an increase in fosfomycin activity, reducing by ≥3 $\log_2$ dilutions the fosfomycin concentration able to eliminate viable bacteria. Despite the fact that the isolates were susceptible (Kp58 and Kp88), but had mutations in GlpT, none of the fosfomycin concentrations tested were able to eradicate bacterial growth. Similar results were found with the highly resistant strains (Kp86 and Kp108). No differences in viability were observed with the addition of PPF against Kp58, Kp86, Kp88, and Kp108.

Time-kill assays. *K. pneumoniae* ATCC 700721, Kp12, Kp142, and their derivatives were evaluated at two concentrations of fosfomycin (64 and 307 mg/liter), with and without 0.623 mM PPF. The results of the time-kill assays are shown in Table 2 and Fig. S3, S4, and S5.

All the tested strains showed the emergence of bacterial subpopulations able to

Downloaded from http://aac.asm.org/ on March 5, 2021 at USE/BCTA.GEN UNIVERSITARIA

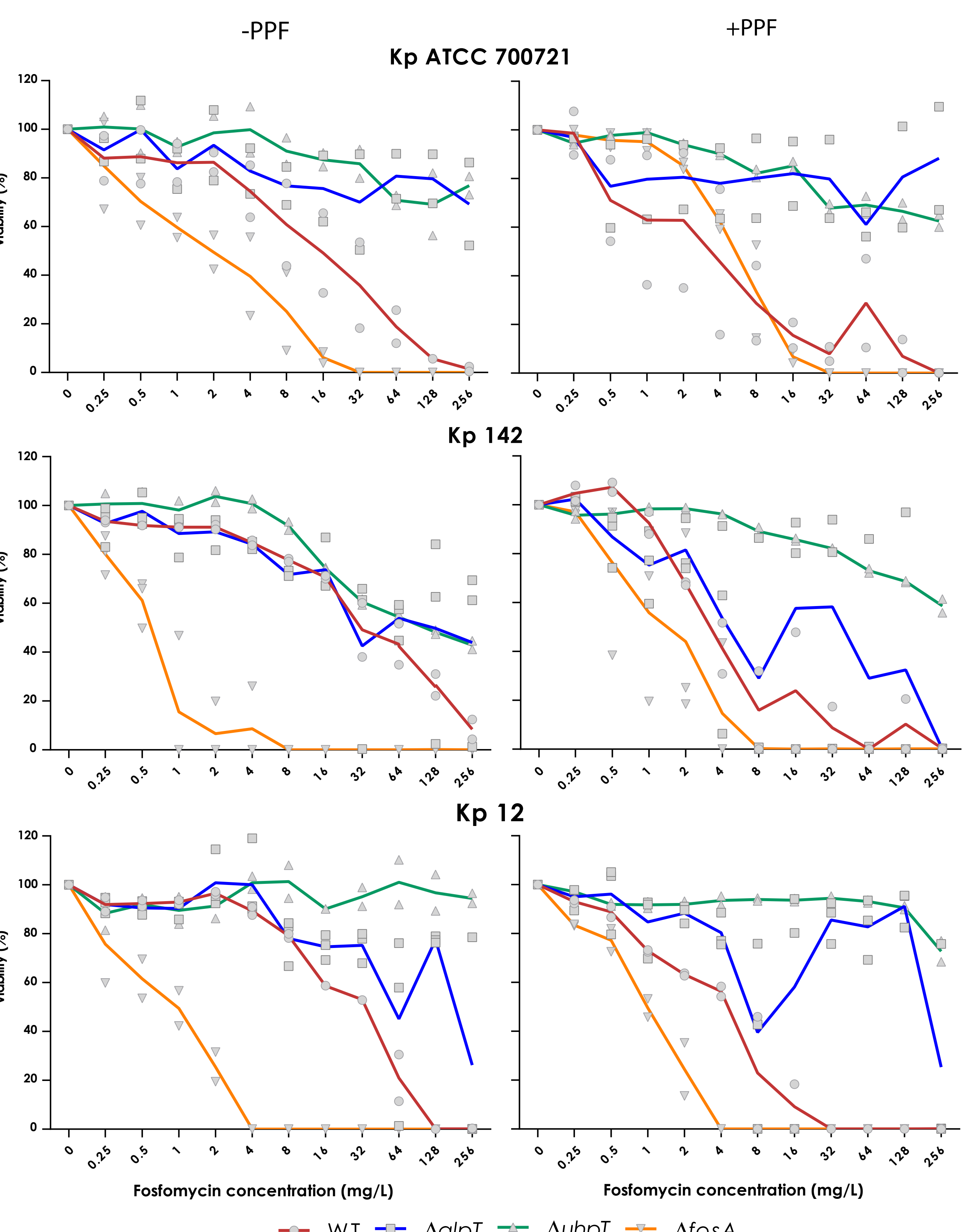


FIG 3 Viability of *K. pneumoniae* ATCC 700721, Kp12, and Kp142 wild-type and isogenic mutant strains against fosfomycin concentrations from 0 to 256 mg/liter, with and without 0.623 mM PPF after 24 h. Lines stand for mean values of measured viability. Symbols represent single results of the replicates.

Downloaded from http://aac.asm.org/ on March 5, 2021 at USE/BCTA.GEN UNIVERSITARIA

TABLE 2 Time-kill results for *K. pneumoniae* ATCC 700721, Kp12, and Kp142 wild-type and isogenic mutant strains using 64 and 307 mg/liter of fosfomycin, alone and in combination with 0.623 mM PPF[a]

	Fosfomycin concentration	Time (h)	KpATCC				Kp12				Kp142			
			WT	Δ*fosA*	Δ*glpT*	Δ*uhpT*	WT	Δ*fosA*	Δ*glpT*	Δ*uhpT*	WT	Δ*fosA*	Δ*glpT*	Δ*uhpT*
No PPF	64 mg/L	2	-1.79	-1.16	-1.69	-0.34	-1.43	-2.39	-1.64	0.35	-1.74	-3.48	-3.27	-0.21
		4	-1.40	-1.42	-1.92	-0.41	-2.60	-3.94	-2.28	1.44	-2.75	-4.28	-1.94	1.33
		8	-1.17	-1.82	1.69	0.47	-1.03	-4.84	0.25	2.80	-1.99	-4.13	1.15	2.06
		24	3.37	-2.35	4.12	3.80	3.16	-1.88	3.55	3.26	2.94	-4.04	3.93	3.74
	307 mg/L	2	-1.41	-1.33	-1.66	-1.59	-3.37	-3.06	-3.91	-0.28	-2.59	-2.44	-2.28	-1.85
		4	-2.19	-1.64	-1.95	-1.29	-3.18	-4.08	-2.85	0.84	-3.22	-3.48	-2.05	-2.04
		8	-3.19	-1.94	2.01	0.92	-1.24	-4.08	-0.17	2.65	-3.50	-4.13	1.30	0.59
		24	-0.44	-2.10	4.06	3.95	2.57	-4.08	2.30	2.45	-5.02	-4.13	3.99	3.79
PPF 0.623mM	64 mg/L	2	-2.15	-2.46	-2.47	-0.56	-2.04	-2.79	-1.81	0.64	-1.97	-2.87	-2.25	0.63
		4	-2.60	-4.16	-2.69	0.47	-2.89	-4.33	-2.49	1.50	-2.14	-5.11	-2.47	1.20
		8	-0.80	-1.57	0.76	0.33	-1.64	-3.58	-0.31	2.74	-2.17	-3.14	0.83	2.67
		24	3.62	-0.80	3.81	3.35	3.11	-0.30	3.59	3.32	2.31	0.38	3.83	4.29
	307 mg/L	2	-2.64	-3.53	-1.79	-1.00	-2.50	-2.52	-1.93	-0.31	-2.84	-4.27	-2.16	-0.84
		4	-4.15	-4.96	-2.54	-1.03	-4.15	-5.04	-2.35	0.01	-2.78	-5.27	-2.23	0.28
		8	-3.21	-4.96	0.85	0.47	-3.85	-5.04	-0.03	2.06	-3.08	-5.27	0.71	0.11
		24	0.36	-0.32	3.55	3.92	2.92	-2.43	3.66	3.77	-1.04	-3.72	3.84	6.13

[a]The results are represented as differences (log_{10} CFU per milliliter) relative to the initial time point (0 h). Green indicates a >3-log_{10} CFU/ml decrease, yellow a 3- to 0-log_{10} CFU/ml decrease, and red no bacterial reduction. WT, wild type.

grow at 64 mg/liter fosfomycin in the control tubes (assays without fosfomycin). In all these cases, subpopulations did not displace the main bacterial population. No differences were observed with the addition of PPF.

Fosfomycin (64 mg/liter) showed bactericidal activity (>3-log CFU/ml decrease) against Kp12 Δ*fosA* (at 4 and 8 h), Kp142 Δ*fosA* (from 0 to 24 h), and Kp142 Δ*glpT* (at 2 h). Fosfomycin reduced the bacterial burden of WT and Δ*glpT* strains in the first 8 h and 4 h, respectively. Δ*uhpT* mutants showed mild (*K. pneumoniae* ATCC 700721 and Kp142) or no (Kp12) killing at 64 mg/liter of fosfomycin at the beginning of the assay, in the first 4 h. All the strains in the isogenic collection, except for Δ*fosA* *K. pneumoniae* ATCC 700721 and Kp142, regrew after 24 h at this concentration due to the emergence of resistant subpopulations.

Compared with the assay with 64 mg/liter of fosfomycin alone, the addition of PPF showed bactericidal activity against *K. pneumoniae* ATCC 700721 (Δ*fosA*) at 4 h and regrowth of Kp142 (Δ*fosA*) after 24 h.

After increasing the fosfomycin concentration to 307 mg/liter, only higher activity against WT strains and prevention of bacterial regrowth of Δ*fosA* (Kp12 and Kp142 strains) were observed.

The addition of PPF increased fosfomycin activity at this concentration, with bactericidal activity against the *K. pneumoniae* ATCC 700721 (WT) strain at 4 h and the Kp12 (Δ*fosA*) strain at 2 to 8 h. Kp12 (Δ*fosA*) and Kp142 (WT) showed bacterial regrowth after 24 h relative to the assay without PPF. All the recovered strains showed fosfomycin MICs of >1,024 mg/liter.

DISCUSSION

The spread of multidrug-resistant bacteria is becoming a crucial public health problem (2). Fosfomycin has recently aroused great interest for the treatment of severe infections caused by *K. pneumoniae* (13, 14), although its activity is often limited by multiple mechanisms, including transporter defects, target modifications, and *fosA*-mediated inactivation. The impact of fosfomycin-inactivating enzymes on fosfomycin resistance among *Enterobacteriaceae* has been widely studied from different perspectives (13, 14). In this sense, various studies have looked for compounds able to reduce or inactivate their activity against fosfomycin (15, 16). Nevertheless, the combination of these determinants together with commonly observed mutations affecting fosfomycin intake has not been widely studied.

Downloaded from http://aac.asm.org/ on March 5, 2021 at USE/BCTA.GEN UNIVERSITARIA

The present study evaluated the role of specific fosfomycin resistance determinants present in *K. pneumoniae* and their impact on the emergence of highly resistant strains during *in vitro* fosfomycin exposure. It also investigated a potential therapeutic approach using the FosA inhibitor sodium phosphonoformate, aimed at increasing fosfomycin susceptibility in *K. pneumoniae* clinical isolates.

In our isogenic *K. pneumoniae* collection of fosfomycin resistance-related genes, a baseline fosfomycin MIC of 32 mg/liter was observed for ATCC 700721, Kp12, and Kp142. Inactivation of *glpT* did not increase fosfomycin resistance relative to that of the wild-type strains. This phenotype has previously been observed in *E. coli* due to the use of glucose-6-phosphate in susceptibility assays (1, 17). Under these conditions, *uhpT* expression masks other fosfomycin resistance-related mutations, especially those related to the glycerol-3-phosphate transporter. On the other hand, inactivation of *uhpT* significantly increased the fosfomycin MIC (≥1,024 mg/liter). The likely explanation for the reduced susceptibility is reduced uptake together with fosfomycin inactivation by the chromosomally mediated FosA.

Similar susceptibility results were found among *fosA* mutants. The inactivation of this chromosomal resistance determinant produced a 32-fold reduction in the fosfomycin MIC with respect to that of the wild-type strains. This change in susceptibility matches the modal MIC observed in the EUCAST fosfomycin MIC distribution found in *E. coli* (1 mg/liter and epidemiological cutoff [ECOFF] of 4 mg/liter), which lacks the chromosomal *fosA* gene (9, 18). The addition of PPF increased fosfomycin susceptibility in a variable way, although no activity was found in the *uhpT* mutant. This effect would not be explained by an increase in mutant frequency caused by PPF. Nevertheless, no differences in selection of rifampicin-resistant mutants were observed. Another plausible explanation is that PPF may compete with fosfomycin for transportation via the UhpT transporter, and this effect would be more evident in a strain lacking *fosA*, although this hypothesis was not studied.

With respect to the pharmacological approach for inhibiting FosA activity, a synergy study was performed combining fosfomycin and PPF.

The maximum synergistic area occurred within the fosfomycin susceptibility range (2 to 8 mg/liter for *K. pneumoniae* ATCC 700721 and 8 to 32 mg/liter of fosfomycin for Kp12 and Kp142), using EUCAST susceptibility breakpoints for *E. coli* (19). This synergistic activity was also promoted with the addition of PPF in a range from 0.3 to 2.5 mM, which corresponds to human blood concentrations after administration of 90 mg/kg of body weight every 12 h (q12h) (mean ± standard deviation [SD] for steady-state maximum concentration of drug in serum [C_{max}], 0.623 ± 0.132 mM) (20). Similar results were obtained by Ito et al. using agar dilution assays (12), in which they found an apparent lack of activity of PPF at concentrations of up to 0.667 mM against some clinical strains or transformants with high fosfomycin MIC values.

The growth monitoring assays showed increased fosfomycin activity when *fosA* was absent relative to the wild-type strain. Despite showing better fosfomycin activity with the addition of PPF, these results did not improve those obtained with Δ*fosA* strains. These discrepancies could be explained as the inefficient activity of PPF blocking FosA or the degradation or inactivation of PPF during the assay.

With respect to the Δ*glpT* mutants, fosfomycin activity was lower than for the wild-type strains, despite having similar MICs. These results were similar to those previously obtained by our group in *E. coli*, whereby highly resistant mutants emerged from Δ*glpT* strains following the selection of additional mutations in loci associated with fosfomycin resistance (1). These results also fit the higher fosfomycin mutant frequencies observed in Δ*glpT* strains than in wild-type strains. No improved effects were observed against Δ*uhpT* mutants when the assays were supplemented with PPF.

Comparable results were obtained with the other clinical isolates. The effect of PPF was observed in strains with low MICs and in the absence of fosfomycin resistance-related mutations, whereas a lack of PPF efficacy was observed when fosfomycin MIC was high or Δ*glpT* was present.

Overall, the time-kill assays showed results similar to those found in the growth assays. Inactivation of *fosA* greatly improved fosfomycin efficacy with bacterial

Downloaded from http://aac.asm.org/ on March 5, 2021 at USE/BCTA.GEN UNIVERSITARIA

reductions of between 1 and $5\log_{10}$ CFU/ml. The addition of PPF to the time-kill assays improved fosfomycin activity only in the first hours, but the emergence of highly resistant mutants occurred in all assays. Our study differs slightly from the results obtained by Ito et al. (12). In that study, using a single *K. pneumoniae* strain with fosfomycin MIC of 256 mg/liter, the authors found a bactericidal effect at 8 and 24 h at concentrations of 256 and 512 mg/liter but bacterial regrowth at 24 h with 128 mg/liter. The addition of PPF to this assay showed a bactericidal effect at all concentrations of fosfomycin tested, including 128 mg/liter.

In conclusion, strategies aimed at inactivating *fosA* activity through gene editing (21) or using pharmacological approaches are promising for increasing fosfomycin activity against *K. pneumoniae* strains. In this respect, the pharmacological route is the most plausible one, but further PK/PD models should be carried out to better assess the activity of FosA-inactivating compounds.

MATERIALS AND METHODS

Bacterial strains. Seven clinical multidrug-resistant *K. pneumoniae* strains from the Andalusian Reference Laboratory for Molecular Typing of Nosocomial Pathogens (PIRASOA program) categorized as susceptible or resistant to fosfomycin by MicroScan WalkAway (Beckman Coulter, Indianapolis, IN) were used. *K. pneumoniae* ATCC 700721 was used as the reference strain.

The two clinical isolates (Kp12 and Kp142) with the lowest fosfomycin MIC values and the reference strain *K. pneumoniae* ATCC 700721 were selected for isogenic mutant construction.

Whole-genome sequencing. The 7 clinical isolates were subjected to whole-genome sequencing on the MiSeq platform (Illumina, San Diego, CA), and sequencing was performed in-house. For this, the libraries were prepared with the Nextera XT DNA library preparation kit, and sequencing was done with a V3 600-cycle reagent cartridge. Sequencing was achieved with at least 30× average coverage. Illumina sequences were assembled *de novo* using the CLC genomics Workbench (Qiagen, Netherlands). The genomes were annotated with Rapid Annotation using Subsystem Technology (RAST) (22). The fosfomycin resistance-related proteins (MurA, GlpT, UhpT, FosA, CyaA, Crp, PtsI, UhpA, UhpB, and UhpC) were compared with *K. pneumoniae* ATCC 700721 using the NCBI BLAST online application.

Isogenic mutant construction. Δ*fosA*, Δ*glpT*, and Δ*uhpT* mutants were constructed using the λ-red gene replacement method, described by Huang et al. (23). All the required primers and plasmids are listed in Table S1. Briefly, the genes of interest (*fosA*, *glpT*, and *uhpT*) were removed by homologous recombination using an apramycin resistance cassette flanked by two 60-bp homologous sequences of the desired gene using the L-arabinose (Sigma-Aldrich, Madrid, Spain)-inducible recombinase of the pACBSR-Hyg plasmid. FLP recombination target (FRT) sites were also included in the construction to remove the apramycin resistance cassette (using pFLP-Hyg) from the chromosome once the mutants had been constructed. All the mutants were confirmed by PCR with sequence-specific primers for each region.

Susceptibility testing. Fosfomycin *in vitro* activity was determined by the gradient strip method (Liofilchem Diagnostici, Italy) using Mueller-Hinton agar (MHA). EUCAST fosfomycin susceptibility breakpoints for *Enterobacterales* were used (19).

In addition, fosfomycin susceptibility testing was performed in Mueller-Hinton agar containing 0.623 mM PPF (Clinigen Healthcare, Staffordshire, UK). This concentration was selected on the basis of the maximum synergy results obtained in the synergy assay and corresponding to the C_{max} values for dosing of 90 mg/kg q12h (20). Assays were performed in duplicate.

Synergy assay. Synergies between fosfomycin and PPF were studied against Kp12, Kp142, and *K. pneumoniae* ATCC 700721 using the checkerboard assay. Briefly, the synergy assay was performed with an inoculum of 5×10^5 CFU/ml in 96-well plates with a final volume of 100 μl per well. Assays were conducted using Mueller-Hinton broth (MHB) supplemented with 25 mg/liter of glucose-6-phosphate. Fosfomycin concentrations ranged from 0.5 to 512 mg/liter and PPF concentrations from 0.16 to 10 mM. Wells without fosfomycin or PPF were used as single-drug assays or growth controls. Bacterial densities were quantified spectrophotometrically by measuring optical density (OD) at 595 nm using an Infinite200 Pro plate reader at 24 h. Bacterial viability was calculated as the ratio of final bacterial OD to the final bacterial OD of the control growth well (without drug). A four-parameter log logistic model was fitted to the data to generate dose-response curves for fosfomycin and PPF. Drug combination responses were also plotted as heat maps to assess the therapeutic significance of the combination by identifying the concentrations at which the drug combination had maximum effect on bacterial growth inhibition. The degree of drug synergy over the full dose-response matrix was analyzed using the response surface model, zero interaction potency (ZIP) with the SynergyFinder package for R (24). Synergy assays were performed in triplicate. The summary synergy represents the average excess response due to drug interactions. A synergy score of less than −10 was considered antagonistic, a range from −10 to +10 as additive, and greater than +10 as synergistic (25).

Mutant frequencies. Fosfomycin-resistant mutant frequencies were assessed for *K. pneumoniae* ATCC 700721, Kp12, Kp142, and their isogenic mutants. As PPF is a pyrophosphate analogue, its role in interfering with the replication process was assessed by analyzing the frequency of rifampicin-resistant

Downloaded from http://aac.asm.org/ on March 5, 2021 at USE/BCTA.GEN UNIVERSITARIA

spontaneous mutants in wild-type *K. pneumoniae* and *E. coli* MG1655 strains (as a control due to the absence of chromosomal *fosA*).

Mutant frequencies were determined in triplicate as previously described (1). Fosfomycin-resistant mutants were recovered in MHA plates supplemented with 25 mg/liter of glucose-6-phosphate (Sigma-Aldrich) and two concentrations of fosfomycin (64 and 512 mg/liter; Sigma-Aldrich). Rifampicin-resistant mutants were recovered after overnight incubation of the bacterial culture with and without PPF (0.623 mM) and then plated on MHA with 100 mg/liter of rifampicin (Sigma-Aldrich). Mutant frequencies were the ratio of mutant to total CFU.

Bacterial growth monitoring. Bacterial growth curves were performed with 5×10^5 CFU/ml as the starting inoculum, using 96-well plates (Nunclon Delta Surface; Thermo Scientific, MA) with a 200-μl volume per well. Assays were conducted using MHB supplemented with 25 mg/liter glucose-6-phosphate, with or without 0.623 mM PPF. Fosfomycin concentrations ranged from 0.25 to 256 mg/liter. Bacterial growth was quantified spectrophotometrically (595 nm) every hour, for 24 h, with an Infinite200 Pro plate reader (Tecan Group AG, Männedorf, Switzerland). Assays were performed in triplicate.

Time-kill assays. Time-kill assays were conducted in duplicate using fosfomycin concentrations of 0 (as a growth control), 64, and 307 mg/liter (maximal concentration of fosfomycin in plasma after intravenous administration of 8 g q8h) (26) with 25 mg/liter of glucose-6-phosphate, with or without 0.623 mM PPF. Briefly, single overnight colonies of each strain were used to prepare the preinoculum in Mueller-Hinton II broth and incubated overnight with shaking at 37°C. The starting inoculum was set at 5×10^5 CFU/ml in a final volume of 20 ml, and bacterial cultures were incubated at 37°C with shaking. The number of viable CFU was determined at 0, 2, 4, 8, and 24 h by serial dilution, followed by plating on MHA plates with or without 64 mg/liter fosfomycin and 25 mg/liter of glucose-6-phosphate. The number of colonies was counted after 24 h of incubation.

When bacterial regrowth was observed after 24 h, up to 3 colonies were selected to assess fosfomycin MIC gradient strips. The survivors were serially passaged three times on fosfomycin-free plates to assess the stability of the phenotype and the fosfomycin MIC was determined, as previously described.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.

ACKNOWLEDGMENTS

Miriam Ortiz-Padilla is supported by a PFIS fellowship from Instituto de Salud Carlos III. This study was supported by the Plan Nacional de I+D+i 2013-2016 and Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Economía, Industria y Competitividad, Spanish Network for Research in Infectious Diseases (PI16/01824, REIPI RD12/0015/0010, and REIPI RD16/0016/0001), cofinanced by European Development Regional Fund A Way To Achieve Europe, Operative Program Intelligent Growth 2014-2020, and the Consejería de Igualdad, Salud y Políticas Sociales, Junta de Andalucía (PI-0044 to 2013).

The funders had no role in the design, collection of data, analysis and writing of the manuscript, or the decision to publish.

J.R.-B. has been scientific coordinator of a research project unrelated to the project funded by AstraZeneca and a speaker at accredited educational activities funded by Merck through unrestricted grants. J.R.-B. and A.P. received funding in kind for research from COMBACTE-NET (grant agreement 115523), COMBACTE-CARE (grant agreement 115620), and COMBACTE-MAGNET (grant agreement 115737) projects under the Innovative Medicines Initiative (IMI), the European Union and EFPIA companies.

REFERENCES

1. Ballestero-Téllez M, Docobo-Pérez F, Portillo-Calderón I, Rodríguez-Martínez JM, Racero L, Ramos-Guelfo MS, Blázquez J, Rodríguez-Baño J, Pascual A. 2017. Molecular insights into fosfomycin resistance in Escherichia coli. J Antimicrob Chemother 72:1303–1309. https://doi.org/10.1093/jac/dkw573.
2. Prestinaci F, Pezzotti P, Pantosti A. 2015. Antimicrobial resistance: a global multifaceted phenomenon. Pathog Glob Health 109:309–318. https://doi.org/10.1179/2047773215Y.0000000030.
3. Docobo-Pérez F, Drusano GL, Johnson A, Goodwin J, Whalley S, Ramos-Martín V, Ballestero-Tellez M, Rodriguez-Martinez JM, Conejo MC, Van Guilder M, Rodríguez-Baño J, Pascual A, Hope WW. 2015. Pharmacodynamics of fosfomycin: insights into clinical use for antimicrobial resistance. Antimicrob Agents Chemother 59:5602–5610. https://doi.org/10.1128/AAC.00752-15.
4. Falagas ME, Maraki S, Karageorgopoulos DE, Kastoris AC, Mavromanolakis E, Samonis G. 2010. Antimicrobial susceptibility of multidrug-resistant (MDR) and extensively drug-resistant (XDR) Enterobacteriaceae isolates to fosfomycin. Int J Antimicrob Agents 35:240–243. https://doi.org/10.1016/j.ijantimicag.2009.10.019.
5. Pitout JD, Laupland KB. 2008. Extended-spectrum β-lactamase-producing Enterobacteriaceae: an emerging public-health concern. Lancet Infect Dis 8:159–166. https://doi.org/10.1016/S1473-3099(08)70041-0.
6. Veve MP, Wagner JL, Kenney RM, Grunwald JL, Davis SL. 2016. Comparison of fosfomycin to ertapenem for outpatient or step-down therapy of extended-spectrum β-lactamase urinary tract infections. Int J Antimicrob Agents 48:56–60. https://doi.org/10.1016/j.ijantimicag.2016.04.014.
7. Castañeda-García A, Blázquez J, Rodríguez-Rojas A. 2013. Molecular

Downloaded from http://aac.asm.org/ on March 5, 2021 at USE/BCTA.GEN UNIVERSITARIA

mechanisms and clinical impact of acquired and intrinsic fosfomycin resistance. Antibiotics (Basel) 2:217–236. https://doi.org/10.3390/antibiotics2020217.
8. Marger MD, Saier MH. 1993. A major superfamily of transmembrane facilitators that catalyse uniport, symport and antiport. Trends Biochem Sci 18:13–20. https://doi.org/10.1016/0968-0004(93)90081-W.
9. Ito R, Mustapha MM, Tomich AD, Callaghan JD, McElheny CL, Mettus RT, Shanks RMQ, Sluis-Cremer N, Doi Y. 2017. Widespread fosfomycin resistance in gram-negative bacteria attributable to the chromosomal fosA gene. mBio 8:e00749-17. https://doi.org/10.1128/mBio.00749-17.
10. Li YY, Zheng B, Li YY, Zhu S, Xue F, Liu J. 2015. Antimicrobial susceptibility and molecular mechanisms of fosfomycin resistance in clinical Escherichia coli isolates in mainland China. PLoS One 10:e0135269. https://doi.org/10.1371/journal.pone.0135269.
11. Nakamura G, Wachino JI, Sato N, Kimura K, Yamada K, Jin W, Shibayama K, Yagi T, Kawamura K, Arakawa Y. 2014. Practical agar-based disk potentiation test for detection of fosfomycin-nonsusceptible Escherichia coli clinical isolates producing glutathione S-transferases. J Clin Microbiol 52:3175–3179. https://doi.org/10.1128/JCM.01094-14.
12. Ito R, Tomich AD, McElheny CL, Mettus RT, Sluis-Cremer N, Doi Y. 2017. Inhibition of fosfomycin resistance protein FosA by phosphonoformate (foscarnet) in multidrug-resistant gram-negative pathogens. Antimicrob Agents Chemother 61. https://doi.org/10.1128/AAC.01424-17.
13. Abbott IJ, Meletiadis J, Belghanch I, Wijma RA, Kanioura L, Roberts JA, Peleg AY, Mouton JW. 2018. Fosfomycin efficacy and emergence of resistance among Enterobacteriaceae in an in vitro dynamic bladder infection model. J Antimicrob Chemother 73:709–719. https://doi.org/10.1093/jac/dkx441.
14. Elliott ZS, Barry KE, Cox HL, Stoesser N, Carroll J, Vegesana K, Kotay S, Sheppard AE, Wailan A, Crook DW, Parikh H, Mathers AJ. 2019. The role of fosA in challenges with fosfomycin susceptibility testing of multispecies Klebsiella pneumoniae carbapenemase-producing clinical isolates. J Clin Microbiol 57:e00634-19. https://doi.org/10.1128/JCM.00634-19.
15. Thirumal Kumar D, Lavanya P, George Priya Doss C, Tayubi IA, Naveen Kumar DR, Francis Yesurajan I, Siva R, Balaji V. 2017. A molecular docking and dynamics approach to screen potent inhibitors against fosfomycin resistant enzyme in clinical Klebsiella pneumoniae. J Cell Biochem 118:4088–4094. https://doi.org/10.1002/jcb.26064.
16. Tomich AD, Klontz EH, Deredge D, Barnard JP, McElheny CL, Eshbach ML, Weisz OA, Wintrode P, Doi Y, Sundberg EJ, Sluis-Cremer N. 2019. Small-molecule inhibitor of FosA expands fosfomycin activity to multidrug-resistant Gram-negative pathogens. Antimicrob Agents Chemother 63: e01524-18. https://doi.org/10.1128/AAC.01524-18.
17. Portillo-Calderón I, Ortiz-Padilla M, Rodríguez-Martínez JM, de Gregorio-Iaria B, Blázquez J, Rodríguez-Baño J, Pascual A, Docobo-Pérez F. 2020. Contribution of hypermutation to fosfomycin heteroresistance in Escherichia coli. J Antimicrob Chemother 75:2066–2075. https://doi.org/10.1093/jac/dkaa131.
18. European Committee on Antimicrobial Susceptibility Testing. 2020. Antimicrobial wild type distributions of microorganisms. https://mic.eucast.org/Eucast2/.
19. The European Committee on Antimicrobial Susceptibility Testing. 2020. Breakpoint tables for interpretation of MICs and zone diameters. Version 10.0. http://www.eucast.org.
20. Spanish Agency of Medicines and Medical Devices (Agencia española del medicamento y productos sanitarios, AEMPS). 2020. Ficha técnica: foscavir 24 mg/ml solución para perfusión. Agencia Española de Medicamentos y Productos Sanitarios (AEMPS), Madrid, Spain. https://cima.aemps.es/cima/pdfs/es/ft/59712/FT_59712.pdf.
21. Kim J-S, Cho D-H, Park M, Chung W-J, Shin D, Ko KS, Kweon D-H. 2016. CRISPR/Cas9-mediated re-sensitization of antibiotic-resistant Escherichia coli harboring extended-spectrum β-lactamases. J Microbiol Biotechnol 26:394–401. https://doi.org/10.4014/jmb.1508.08080.
22. Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, Formsma K, Gerdes S, Glass EM, Kubal M, Meyer F, Olsen GJ, Olson R, Osterman AL, Overbeek RA, McNeil LK, Paarmann D, Paczian T, Parrello B, Pusch GD, Reich C, Stevens R, Vassieva O, Vonstein V, Wilke A, Zagnitko O. 2008. The RAST Server: Rapid Annotations using Subsystems Technology. BMC Genomics 9:75. https://doi.org/10.1186/1471-2164-9-75.
23. Huang TW, Lam I, Chang HY, Tsai SF, Palsson BO, Charusanti P. 2014. Capsule deletion via a λ-Red knockout system perturbs biofilm formation and fimbriae expression in Klebsiella pneumoniae MGH 78578. BMC Res Notes 7:13. https://doi.org/10.1186/1756-0500-7-13.
24. Yadav B, Wennerberg K, Aittokallio T, Tang J. 2015. Searching for drug synergy in complex dose-response landscapes using an interaction potency model. Comput Struct Biotechnol J 13:504–513. https://doi.org/10.1016/j.csbj.2015.09.001.
25. Ianevski A, Giri AK, Aittokallio T. 2020. SynergyFinder 2.0: visual analytics of multi-drug combination synergies. Nucleic Acids Res 48:W488–W493. https://doi.org/10.1093/nar/gkaa216.
26. Pfausler B, Spiss H, Dittrich P, Zeitlinger M, Schmutzhard E, Joukhadar C. 2004. Concentrations of fosfomycin in the cerebrospinal fluid of neurointensive care patients with ventriculostomy-associated ventriculitis. J Antimicrob Chemother 53:848–852. https://doi.org/10.1093/jac/dkh158.

Downloaded from http://aac.asm.org/ on March 5, 2021 at USE/BCTA.GEN UNIVERSITARIA

Supplementary Material of Interplay among Different Fosfomycin Resistance Mechanisms in *Klebsiella pneumoniae.*

Table S1. Primers and plasmids used to construct and check the mutant isogenic collection.

Primers	Sequence
ApmR-5ext	5'- CTGTTGCAAATAGTCGGTGGTGA-3'
ApmR-3ext	5' -CCGAGATCCGTTGATCTTCCT-3'
glpT_KO_'Fwd'	5'- TTAAGTATTTTAAACCCGCCGCGCATAAAGCGCGTTTGCCA GCAGCGGAGATTGATCCGCTATATCGCCGATTACGCTGattccgg ggatccgtcgacc-3'
glpT_'KO_Rev	5'- GGCTTGTTTCAGCTGTTGGTGATGGCGACGTTCACCGAGCAT CACGATAACCAGCAGGATAACCGCCAGCACGCTGCCGCtgtagg ctggagctgcttc-3'
Pre-H1-glpT_Kp	5'-CACGAAATCACATAACACGCTC-3'
Post-H2-glpT_Kp	5'-CACCAGGTCTTGCTCAAGGTAATC-3'
uhpT_KO_'Fwd'	5'- CTGGCCTTTCTGAATCAGGTGCGCAAGCCGACCCTGGATCTG CCGCTCGATGTGCGGCGCAAAATGTGGTTCAAACCTTTattccgg ggatccgtcgacc-3'
uhpT_'KO_Rev	5'- GGCGGCCTGCAAAATACGGTTCTTTTCTCGCGGCGAATTTTA CGCTCTTCAAAGATAGCCACGATGGCCATCAGGCAGAtgtaggct ggagctgcttc-3'
Pre-H1-uhpT_Kp	5'-CCAGCGAAGCGTGATGTAGCT-3'
Post-H2-uhpT_Kp	5'-CCTCTCATCCAATCGCAGCCTGA-3'
fosA_KO_'Fwd'	5'- CTGAGTGGACTGAATCACCTGACCCTGGCAGTCAGCCAGCTG GCGCCGAGCGTGGCGTTTTATCAGCAGCTGCTGGGCATattccgg ggatccgtcgacc-3'
fosA_'KO_Rev	5'- CTGATCAAAAAACACCATCCCCTTATACGGCTGTTCGCGGCA GGCGGCCAGACGCTGGGCGAGACTGCCGACGTGCAGCTtgtagg ctggagctgcttc-3'
Pre-H1-fosA_Kp	5'-GAGACCCTGCGCAATAGCGAT-3'
Post-H2-fosA_Kp	5'-CCTGCCGCCGACCGAGAAACAT-3'

Plasmid	Description	Source
pIJ773	Template to amplify the apramycin resistance gene, *aac(3)IV*, and flanking FRT sites.	Addgene
pACBSR-Hyg	A p15A replicon plasmid containing an arabinose-inducible λ-red recombinase and hygromycin resistance marker. Thermosensitive; it replicates at 30º but not 37ºC.	Addgene
pFLP-Hyg	Plasmid bearing a heat-shock inducible FLP recombinase with p15A replicon and hygromycin resistance. It was used to excise the apramycin selection marker from the chromosome via FRT sites.	Addgene

Table S2. Median (IQR) frequencies of spontaneous fosfomycin (64 and 512 mg/L) and rifampicin resistant mutants (100 mg/L), with and without the addition of 0.623mM of PPF.

°Strains	Fosfomycin mutant frequencies		Rifampicin mutant frequencies	
	Ø		Ø	+ 0.623mM PPF
	64 mg/L	512 mg/L	100 mg/L	
E. coli MG1655	$<10^{-9}$	$<10^{-9}$	$1.77x10^{-8}$ ($1.15x10^{-8}$, $2.47x10^{-8}$)	$2.09x10^{-8}$ ($1.71x10^{-8}$, $2.46x10^{-8}$)
ATCC 700721	$7.50x10^{-6}$ ($6.25x10^{-6}$, $1.87x10^{-5}$)	$9.29x10^{-7}$ ($9.14x10^{-7}$, $1.09x10^{-6}$)	$3.45x10^{-8}$ ($2.15x10^{-8}$, $3.94x10^{-8}$)	$2.35x10^{-8}$ ($1.06x10^{-8}$, $2.45x10^{-7}$)
ATCC Δ*fosA*	$2.00x10^{-7}$ ($1.50 x10^{-7}$, $1.67 x10^{-6}$)	$5.00x10^{-9}$ ($3.75x10^{-9}$, $5.86x10^{-7}$)	ND	ND
ATCC Δ*glpT*	$1.60x10^{-5}$ ($8.10x10^{-6}$, $2.80x10^{-5}$)	$7.20x10^{-6}$ ($5.35x10^{-6}$, $1.36 x10^{-5}$)	ND	ND
ATCC Δ*uhpT*	1.42 ($8.55 x10^{-1}$, 2.01)	$3.66x10^{-1}$ ($1.83x10^{-1}$, $4.04x10^{-1}$)	ND	ND
Kp12	$2.41x10^{-6}$ ($2.04 x10^{-6}$, $2.89 x10^{-6}$)	$4.42x10^{-7}$ ($2.48x10^{-7}$, $9.26x10^{-7}$)	$5.89x10^{-9}$ ($9.43x10^{-10}$, $8.82x10^{-9}$)	$1.05x10^{-8}$ ($9.26x10^{-10}$, $2.01x10^{-8}$)
Kp12 Δ*fosA*	$4.08x10^{-7}$ ($2.94 x10^{-7}$, $7.09 x10^{-7}$)	$2.98x10^{-10}$ ($2.53x10^{-10}$, $2.98x10^{-10}$)	ND	ND
Kp12 Δ*glpT*	$1.90x10^{-5}$ ($1.09 x10^{-5}$, $3.33 x10^{-5}$)	$6.73x10^{-6}$ ($5.72x10^{-6}$, $2.70x10^{-5}$)	ND	ND
Kp12 Δ*uhpT*	$9.37x10^{-2}$ ($6.49 x10^{-2}$, $1.32 x10^{-1}$)	$9.11x10^{-2}$ ($5.26x10^{-2}$, $1.17x10^{-1}$)	ND	ND
Kp142	$1.04x10^{-4}$ ($5.69 x10^{-5}$, $3.80 x10^{-3}$)	$7.81x10^{-5}$ ($3.9x10^{-5}$, $3.79x10^{-3}$)	$1.32x10^{-8}$ ($6.51x10^{-9}$, $2.23x10^{-8}$)	$2.48x10^{-8}$ ($1.95x10^{-8}$, $3.23x10^{-8}$)
Kp142 Δ*fosA*	$1.67x10^{-9}$ ($1.46 x10^{-9}$, $7.50 x10^{-5}$)	$1.67x10^{-9}$ ($1.46x10^{-9}$, $2.50x10^{-5}$)	ND	ND
Kp142 Δ*glpT*	$7.50x10^{-5}$ ($4.78 x10^{-5}$, 3.00)	$6.00x10^{-6}$ ($4.50x10^{-6}$, $1.40x10^{-1}$)	ND	ND
Kp142 Δ*uhpT*	$4.00x10^{-1}$ ($3.43 x10^{-1}$, 1.70)	$1.33 x10^{-4}$ ($9.52x10^{-5}$, $3.27x10^{-3}$)	ND	ND

Figure S1. Frequencies of spontaneous rifampicin resistant mutants of EcMG1655, KpATCC, Kp12 and Kp142 wild-type strains after incubation, with and without PPF. Black lines represent the median values of the three replicates.

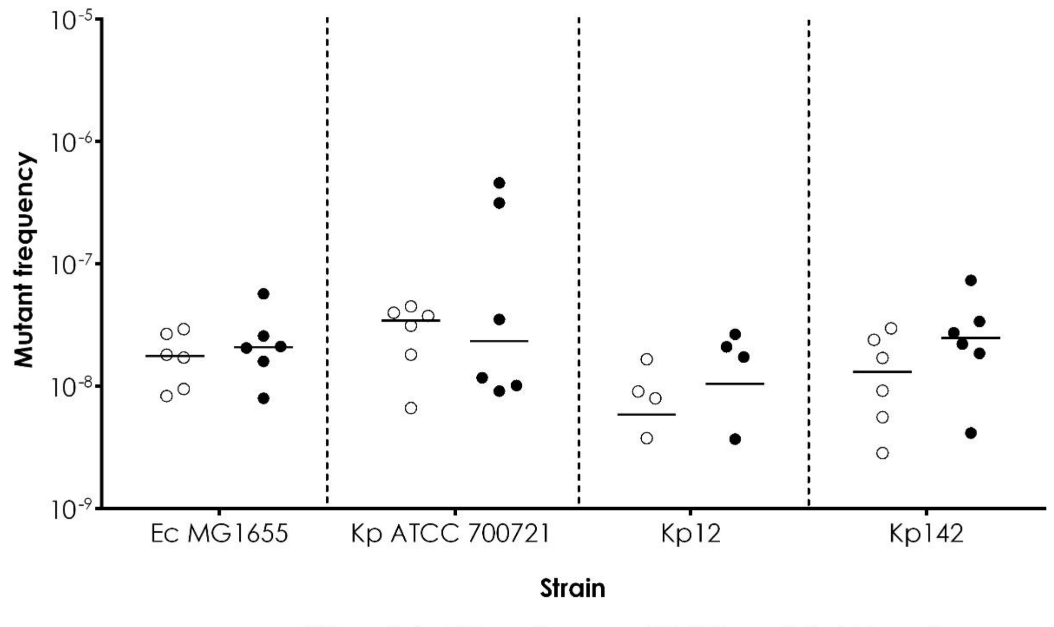


Figure S2. Viability of clinical isolates against fosfomycin concentrations from 0-256 mg/L, with and without 0.623 mM PPF after 24h. Lines stand for mean values of measured viability. Symbols represent single results of the replicates.

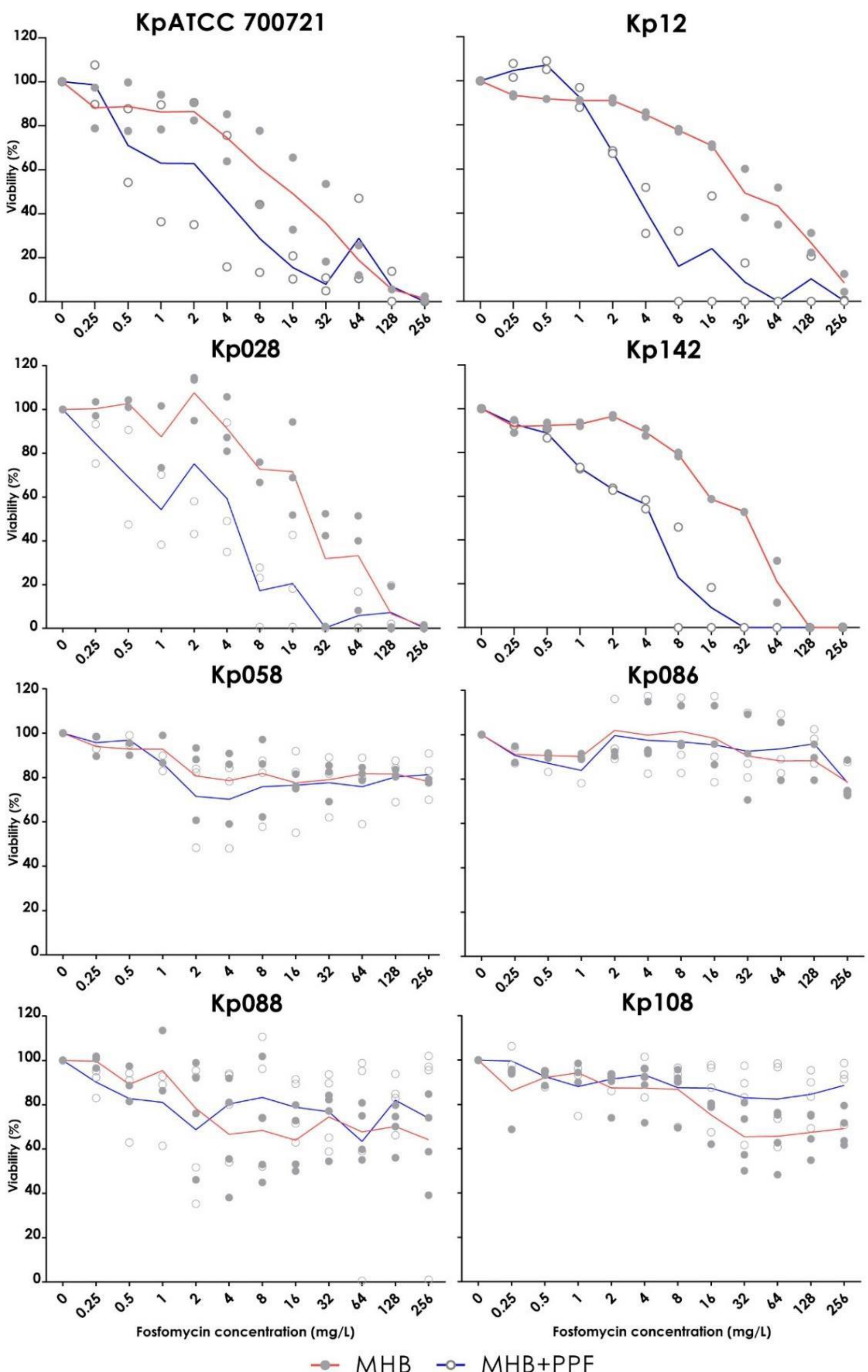


Figure S3. Time-kill results for KpATCC 700721, wild-type (WT), and isogenic mutant strains using 64 and 307 mg/L fosfomycin, alone and in combination with 0.623 mM PPF. Dashed lines represent bacterial concentrations in fosfomycin-containing plates (64 mg/L).

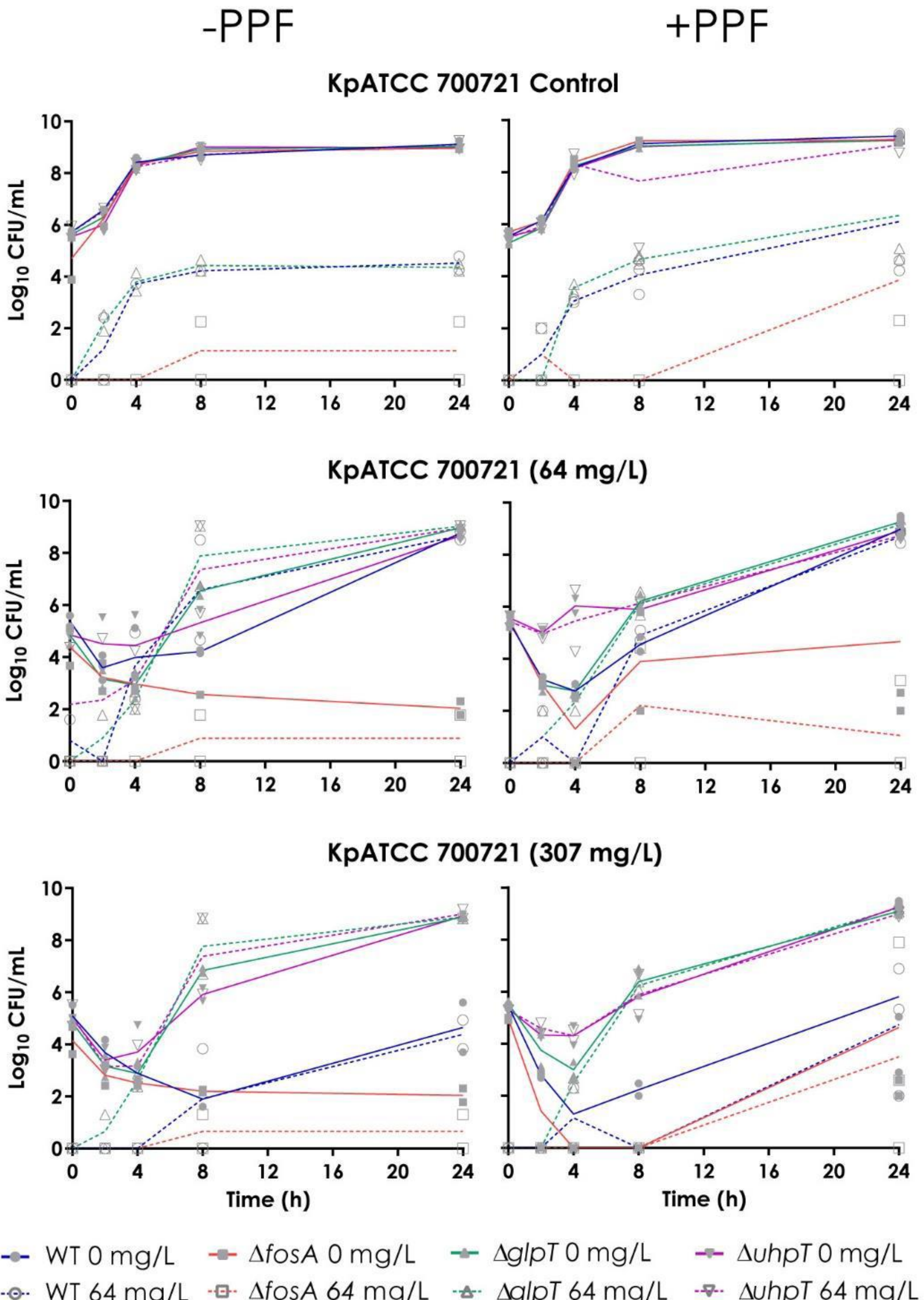


Figure S4. Time-kill results for Kp12, wild-type (WT), and isogenic mutant strains using 64 and 307 mg/L fosfomycin, alone and in combination with 0.623 mM PPF. Dashed lines represent bacterial concentrations in fosfomycin-containing plates (64 mg/L)

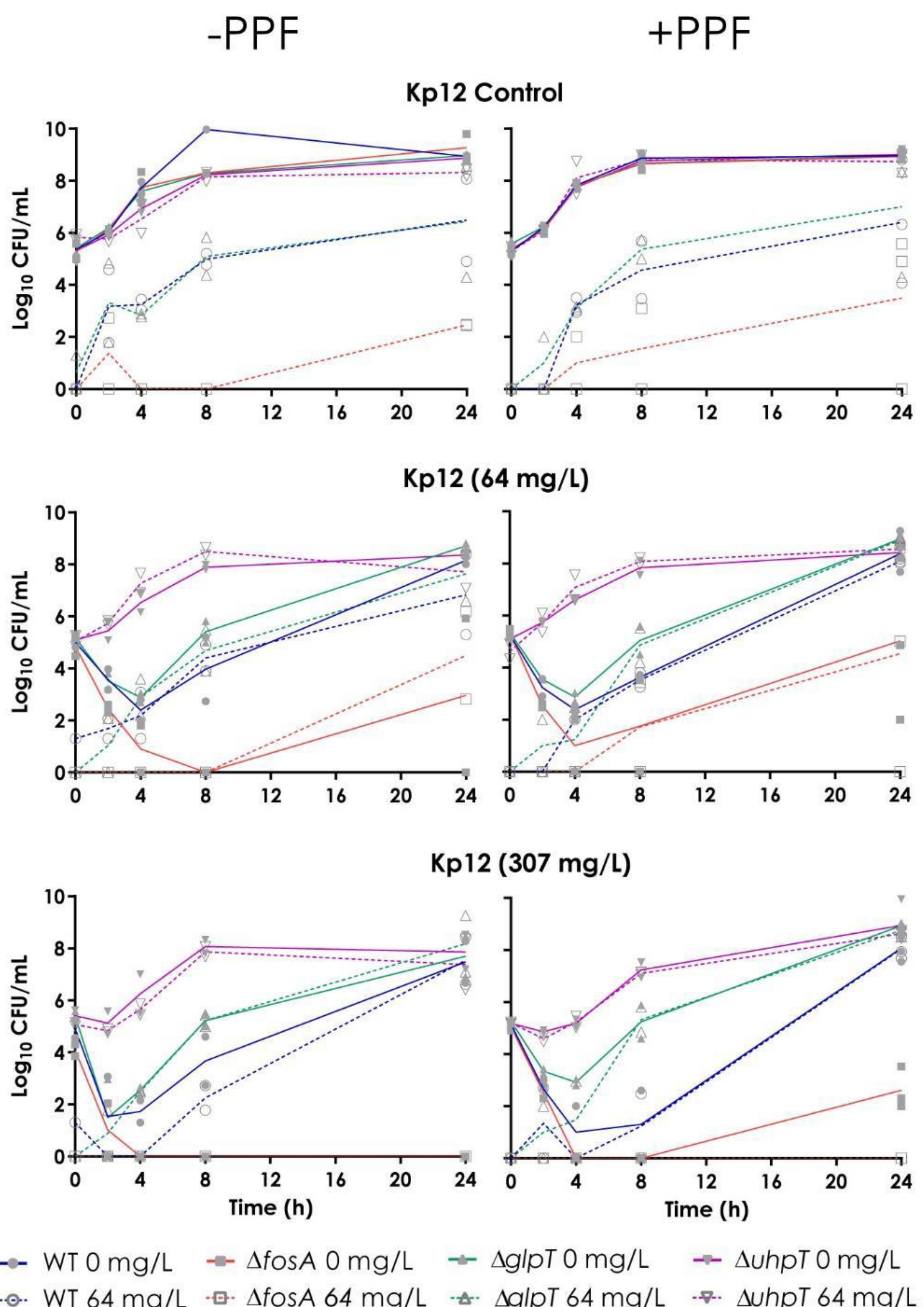
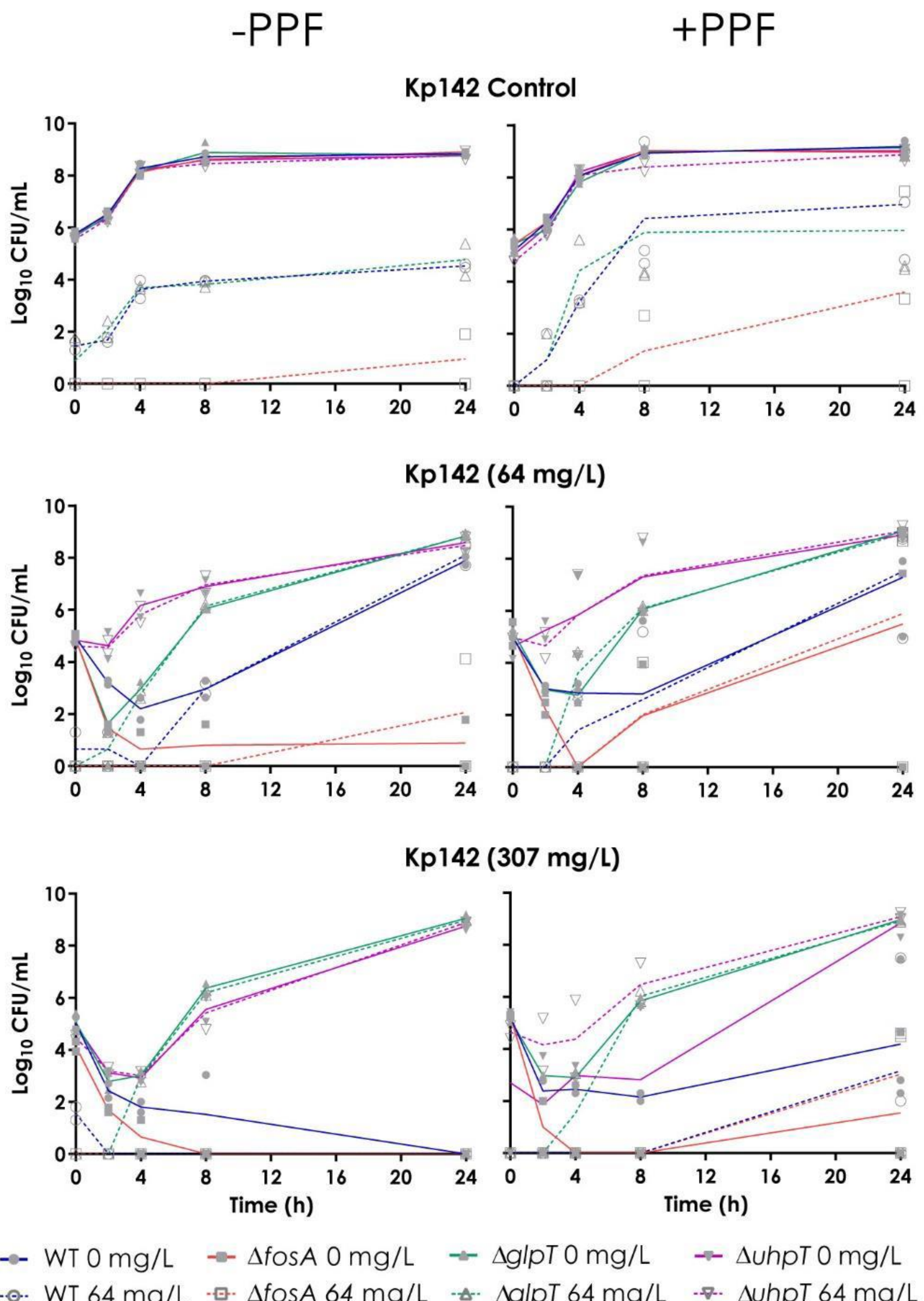


Figure S5. Time-kill results for Kp142, wild-type (WT), and isogenic mutant strains using 64 and 307 mg/L fosfomycin, alone and in combination with 0.623 mM PPF. Dashed lines represent bacterial concentrations in fosfomycin-containing plates (64 mg/L)



2. Effect of Glycerol on Fosfomycin Activity against Escherichia coli.

The bactericidal action of fosfomycin require the entry into the cytoplasm. This entry is mediated by the GlpT (glycerol-3-phosphate transporter) and UhpT transporter (hexose phosphate transporter). UhpT is induced by the presence of G6P, and regarding GlpT, this transporter is induced by the presence of G3P. Thus, to fully observe fosfomycin activity, glucose-6-phosphate (G6P, inductor of UhpT) is added for fosfomycin susceptibility assays according to the CLSI or EUCAST guidelines. However, the role of the GlpT transporter in susceptibility testing remains unclear. Although the addition of glycerol-3-phosphate induces the GlpT transporter, it also reduces the transport of fosfomycin by occupancy of the transporter site.

The use of glycerol could solve the problem of activating the *glpT* transporter (indirect way) without reducing the intake of fosfomycin. However, fosfomycin activity has not been previously studied in combination with glycerol. Therefore, the objective of the present study is to characterize and evaluate the activity of fosfomycin using glycerol at clinically physiological concentrations for the activation of the GlpT transporter.

The results obtained in this work are the following:

Fosfomycin Transporters Promoters Activity

The results on the promoter activity of *glpT* and *uhpT* at the time points 4 h, 12 h, and 24 h are shown in Figure 1 (Art. 2). Furthermore, the 24-h fluorescence kinetics is shown in the Supplementary Materials (Figure S1, Art. 2). The presence of glycerol produced a significant increase in *glpT* transcription for both strains at 12 and 24 h. This increase was minimal or absent within the first 4 h, but after this initial lag period, *glpT* transcription increased during the remainder of the assay. The addition of G6P did not modify the expression of glpT. Regarding the promoter activity of *uhpT*, the addition of G6P, but not glycerol, increased the expression of *uhpT* within the first 4 h, followed by a gradual decline.

The results of flow cytometry are shown in Figure 2 (Art. 2). In the absence of glycerol and G6P, no expression of the *uhpT* gene was observed. The addition of G6P increased the percentage of positive events at 4 h. However, the fluorescence decreased after 24 h. Regarding the expression of the *glpT* gene in MHB alone at 4 h, *E. coli* ATCC25922 showed *glpT* expression. The addition of glycerol increased the percentage of positive events for both strains, except for the case of *E. coli* ATCC25922 with 7 mg/mL of glycerol. At 24 h, almost all the population of *E. coli* ATCC25922 and half of the population of *E. coli* MG1655 showed fluorescence without glycerol. The addition of glycerol increased the percentage of positive events for both strains, except with 7 mg/mL of glycerol. The fluorescence intensity was higher in the case of the *E. coli* ATCC25922 strain, closer to the intensity obtained by the positive control.

Susceptibility Testing

The fosfomycin MICs, performed with the reference method, for the isogenic collection are shown in Table 1 (Art. 2), and the results of disk diffusion are shown in Figure 3 (Art. 2).

With the agar dilution assay, in wild-type strains, the MIC with G6P is 2mg/L, without G6P, the MIC increased to 64 and >64 mg/L. The mutant for *glpR* gen is susceptible in all conditions evaluated (MIC=1-4 mg/L). Mutants for *uhpT* and *glpT-uhpT* presented resistant phenotype in all conditions evaluated (>64 mg/L). The rest of mutant evaluated showed MIC of 1 - 4 mg/L with G6P, and 32 - >64 mg/L. The addition of glycerol did not improve fosfomycin activity (MIC drop by +/-1 log2 dilution) with respect to fosfomycin alone or in combination with G6P, in any conditions evaluated.

Regarding the disk diffusion, in wild-type and mutant Δ*uhpT*, the increase in glycerol causes an increase in the inhibition zone (increased susceptibility). In the mutant Δ*glpT*, there is an increment of inhibition zone with the addition of 1.8 and 7mg/mL of glycerol (no addition of G6P conditions). In the mutant Δ*cyaA*, there is an increment of inhibition zone with the addition of 0.45 and 1.8mg/mL of glycerol (±G6P). The addition of any concentrations of glycerol modification the inhibition zone of the mutants Δ*glpT-uhpT*, Δ*glpK* and Δ*glpR.*

Fosfomycin and Glycerol Interaction Assay

The result of the interaction assay is shown in Table 2 and Figure S2 (Art. 2). The combination of fosfomycin and glycerol showed synergistic activity (ZIP score > +10) G6P for the wild-type strains and for *E. coli* MG1655 Δ*uhpT* strain with G6P and without. The most synergistic area occurred between 7–28 mg/mL of glycerol and 0.5-2 mg/L of fosfomycin for the *E. coli* ATCC25922 strain. Similar results were observed for the *E. coli* MG1655 strain, but at 0.25-1 mg/L of fosfomycin. In the case of MG1655 Δ*uhpT*, the greatest synergy was observed at the same concentration as that observed for the ATCC25922 strain with and without G6P. Antagonism (ZIP score < -10) was observed against the MG1655 Δ*glpT-uhpT* strain under any conditions.

Fosfomycin and Glycerol Time-Kill Assays

The strains were evaluated at two concentrations of fosfomycin (64 and 307 mg/L), without glycerol, and with two concentrations (0.45 and 7 mg/mL). The results are shown in Figure 4 and Figure S3 (Art. 2). The addition of glycerol and G6P did not modify growth under control conditions. Fosfomycin alone at 64 mg/L showed a bactericidal effect within the first 4 h against the wild-type and the *E. coli* MG1655 Δ*uhpT* mutant strains, but bacterial regrowths were observed at 8 and 24 h. The addition of G6P also showed a bactericidal effect within the first 4–8 h in all strains except MG1655 Δ*uhpT* and Δ*glpT-uhpT* and prevented the bacterial regrowth for the wild-type strains and the MG1655 Δ*glpR* and Δ*glpK* mutant strains. The addition of glycerol at both tested concentrations did not improve the bactericidal effect against any of the evaluated strains. Fosfomycin alone at 307mg/L showed a

bactericidal effect against all the strains within the first hours, except for MG1655 Δ*glpT* and Δ*glpT*-*uhpT* strains. However, bacterial regrowth was observed except for the wild-type ATCC25922 and MG1655 Δ*glpR* strains. The addition of G6P improved the bactericidal effect and prevented the regrowth in all the strains except for MG1655 Δ*uhpT* and Δ*glpT*-*uhpT*. The addition of glycerol at both concentrations did not improve the bactericidal effect in any of the evaluated strains. Only against MG1655 Δ*uhpT* strain fosfomycin combined with 0.45 mg/mL of glycerol showed an initial improved activity; however, bacterial regrowth was observed after 24 h.

Article

Effect of Glycerol on Fosfomycin Activity against *Escherichia coli*

Miriam Ortiz-Padilla [1,2,3], Inés Portillo-Calderón [1,3,4], Ana Velázquez-Escudero [1], Jesús Rodríguez-Baño [1,3,4,5], Álvaro Pascual [1,2,3,4], José Manuel Rodríguez-Martínez [2,3,4,*] and Fernando Docobo-Pérez [2,3,4,*]

[1] Unidad Clínica de Enfermedades Infecciosas y Microbiología, Hospital Universitario Virgen Macarena, 41009 Sevilla, Spain
[2] Departamento de Microbiología, Facultad de Medicina, Universidad de Sevilla, 41009 Sevilla, Spain
[3] Instituto de Biomedicina de Sevilla IBIS, Hospital Universitario Virgen Macarena/CSIC/Universidad de Sevilla, 41009 Sevilla, Spain
[4] Centro de Investigación Biomédica en Red en Enfermedades Infecciosas (CIBERINFEC), 28029 Madrid, Spain
[5] Departamento de Medicina, Facultad de Medicina, Universidad de Sevilla, 41009 Sevilla, Spain
* Correspondence: jmrodriguez@us.es (J.M.R.-M.); fdocobo1@us.es (F.D.-P.); Tel.: +34-95-455-6100 (J.M.R.-M. & F.D.-P.)

Abstract: Fosfomycin is an antimicrobial that inhibits the biosynthesis of peptidoglycan by entering the bacteria through two channels (UhpT and GlpT). Glycerol is clinically used as a treatment for elevated intracranial pressure and induces the expression of *glpT* in *Escherichia coli.* Glycerol might offer synergistic activity by increasing fosfomycin uptake. The present study evaluates the use of glycerol at physiological concentrations in combination with fosfomycin against a collection of isogenic mutants of fosfomycin-related genes in *E. coli* strains. Induction of fosfomycin transporters, susceptibility tests, interaction assays, and time-kill assays were performed. Our results support the notion that glycerol allows activation of the GlpT transporter, but this induction is delayed over time and is not homogeneous across the bacterial population, leading to contradictory results regarding the enhancement of fosfomycin activity. The susceptibility assays showed an increase in fosfomycin activity with glycerol in the disk diffusion assay but not in the agar dilution or broth microdilution assays. Similarly, in the time-kill assays, the effect of glycerol was absent by the emergence of fosfomycin-resistant subpopulations. In conclusion, glycerol may not be a good candidate for use as an adjuvant with fosfomycin.

Keywords: fosfomycin; antimicrobial resistance; optimization treatment

Citation: Ortiz-Padilla, M.; Portillo-Calderón, I.; Velázquez-Escudero, A.; Rodríguez-Baño, J.; Pascual, Á.; Rodríguez-Martínez, J.M.; Docobo-Pérez, F. Effect of Glycerol on Fosfomycin Activity against *Escherichia coli*. *Antibiotics* **2022**, *11*, 1612. https://doi.org/10.3390/antibiotics11111612

Academic Editor: Anthony William Coleman

Received: 21 October 2022
Accepted: 10 November 2022
Published: 12 November 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Bacterial resistance has been steadily increasing in recent decades, along with the lack of new active drugs, leading to the need to reuse old antimicrobial agents as an alternative strategy [1,2] to combat increased morbidity and mortality worldwide [3]. Fosfomycin is a broad-spectrum antimicrobial approved for the treatment of uncomplicated urinary tract infections, soft tissue infections, and sepsis caused by Enterobacteriaceae [4,5]. The bactericidal action of fosfomycin is achieved by interrupting the first step of peptidoglycan biosynthesis by blocking the MurA enzyme, thus requiring entry into the cytoplasm. This entry is mediated by the GlpT (glycerol-3-phosphate transporter) and UhpT transporters (hexose phosphate transporter), which belong to the major facilitator superfamily [6]. The expression of these transporters is regulated by many factors. As many other genes involved in the catabolism of secondary carbon sources, both are controlled by the concentration of cyclic AMP (cAMP, synthesized by adenylate cyclase encoded by the *cyaA* gene) bound to its transcriptional dual regulator CRP (cAMP receptor protein receptor) [7]. Therefore, the absence of the *cyaA* gene leads to increased resistance to fosfomycin [8]. Moreover, each transporter is induced by its own substrate. UhpT is induced by the presence of

G6P, which is detected in the periplasmic space by the two-component system UhpBC, where UhpB phosphorylates UhpA acting as a transcriptional activator of *uhpT*. Thus, mutations in any gene in this system lead to increased resistance to fosfomycin [9]. With regard to GlpT, this transporter is induced by the presence of G3P, which binds to GlpR, a transcriptional repressor of *glpT*, causing a loss of affinity for the promoter, thus inducing its transcription. Loss of function of this gene would increase the sensitivity to fosfomycin [10]. In this sense, the main mechanism of resistance in *E. coli* clinical isolates is the loss of function of these transporters or genes involved in their regulation [6]. Thus, to fully observe fosfomycin activity, glucose-6-phosphate (G6P, inductor of UhpT) is added for fosfomycin susceptibility assays according to the CLSI or EUCAST guidelines. However, the role of the GlpT transporter in susceptibility testing remains unclear. Although the addition of glycerol-3-phosphate induces the GlpT transporter, it also reduces the transport of fosfomycin by occupancy of the transporter site [11]. Alternative activators should be explored to overcome this problem in order to increase the activity of fosfomycin. In this sense, glycerol could be a good candidate for this function. Glycerol is a triol that can be used by *E. coli* as a carbon source and has also been used orally or intravenously in clinical practice as a potent osmotic dehydrating agent in the treatment of elevated intracranial pressure [12]. In *E. coli*, glycerol enters the bacterium through passive diffusion or through the glycerol facilitator, the GlpF transporter [13]. Once inside, it is phosphorylated by GlpK kinase, producing intracellular glycerol-3-phosphate. This endogenous glycerol-3-phosphate can remove the GlpR repressor from the *glpT* promoter region, activating its transcription [13–15]. The use of this carbon source could solve the problem of activating the *glpT* transporter without reducing the intake of fosfomycin. However, fosfomycin activity has not been previously studied in combination with glycerol. Therefore, the objective of the present study is to characterize and evaluate the activity of fosfomycin using glycerol at clinically physiological concentrations for the activation of the GlpT transporter.

2. Results

2.1. Fosfomycin Transporters Promoters Activity

The results on the promoter activity of *glpT* and *uhpT* at the time points 4 h, 12 h, and 24 h are shown in Figure 1. Furthermore, the 24-h fluorescence kinetics is shown in the Supplementary Materials (Figure S1). The presence of glycerol produced a significant increase in *glpT* transcription for both strains at 12 and 24 h. This increase was minimal or absent within the first 4 h, but after this initial lag period, *glpT* transcription increased during the remainder of the assay. In the case of *E. coli* ATCC25922, the highest transcription of *glpT* was observed at the maximum glycerol concentration of 7 mg/mL. While in the case of *E. coli* MG1655, the maximum activity of the *glpT* promoter was observed at a glycerol concentration of 1.8 mg/mL. The addition of G6P did not modify the expression of *glpT*. Regarding the promoter activity of *uhpT,* the addition of G6P, but not glycerol, increased the expression of *uhpT* within the first 4 h, followed by a gradual decline.

The results of flow cytometry are shown in Figure 2. In the absence of glycerol and G6P, no expression of the *uhpT* gene was observed for *E. coli* ATCC25922 and *E. coli* MG1655. The addition of G6P increased the percentage of positive events at 4 h (76.9% and 99.7%). However, the fluorescence decreased to 7.7% and 60% after 24 h. Regarding the expression of the *glpT* gene in MHB alone at 4 h, 59.3% of the *E. coli* ATCC25922 population and 1.1% of the *E. coli* MG1655 population showed *glpT* expression. The addition of glycerol increased the percentage of positive events for both strains, except for the case of *E. coli* ATCC25922 with 7 mg/mL of glycerol. At 24 h, almost all the population of *E. coli* ATCC25922 (97.3%) and half of the population of *E. coli* MG1655 (54.5%) showed fluorescence. The addition of glycerol increased the percentage of positive events for both strains, reaching almost 100% of positive events in *E. coli* ATCC25922 and nearly 95% in *E. coli* MG1655, except with 7 mg/mL of glycerol. The fluorescence intensity was higher in the case of the *E. coli* ATCC25922 strain, closer to the intensity obtained by the positive control (pMS201-P*cyaA*::*gfp*mut2).

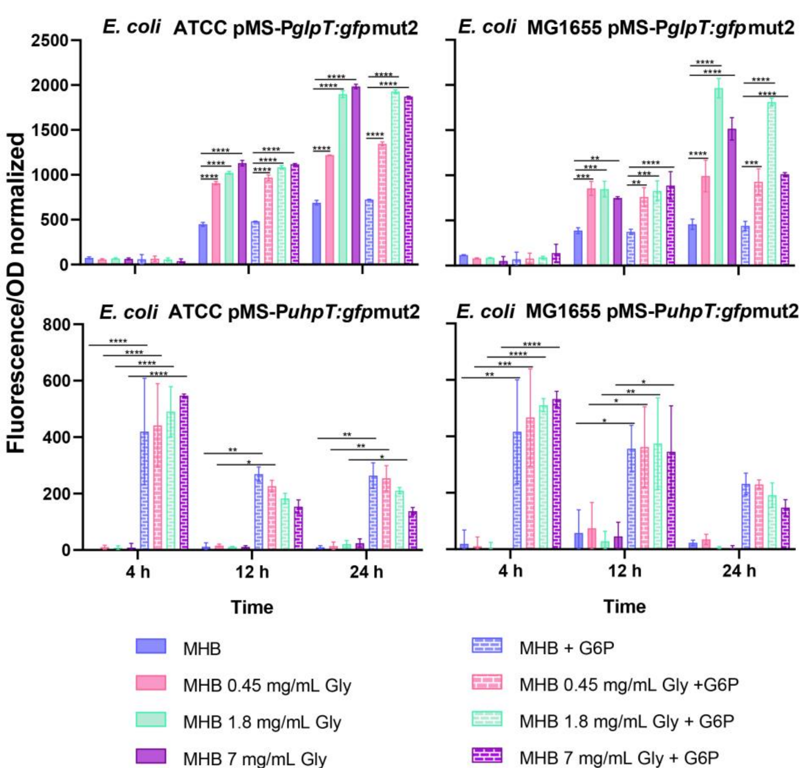
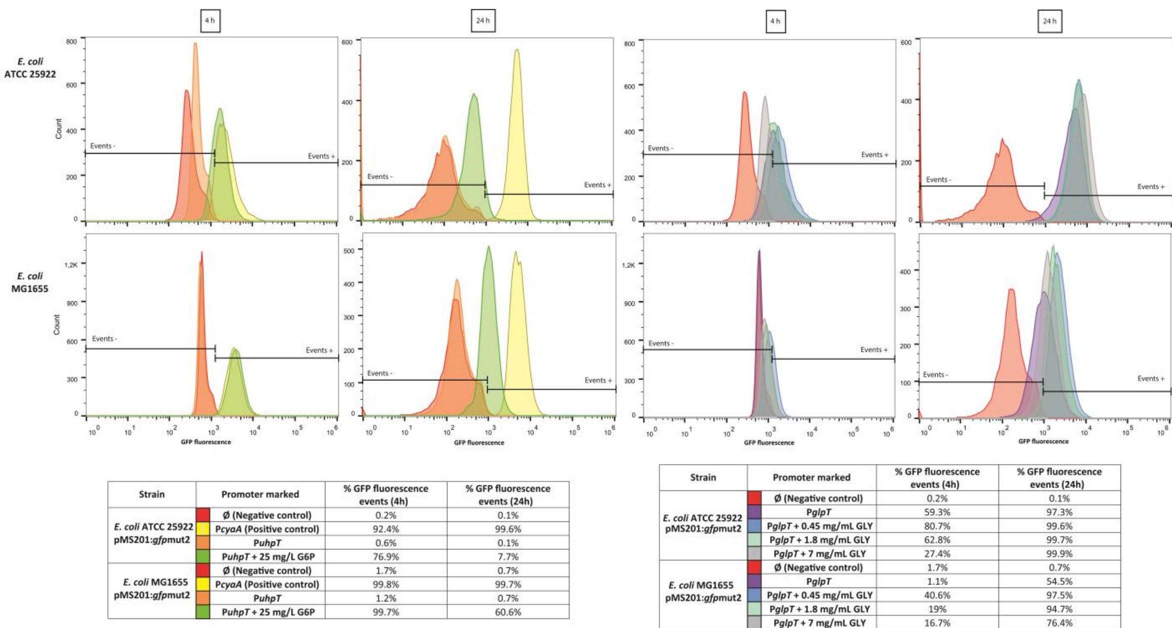


Figure 1. Induction of the *glpT* and *uhpT* genes in the ATCC25922 and MG1655 strains. Fold induction is GFP fluorescence after 4, 12, and 24 h of exposure, normalized to promoterless strains. Error bars represent standard deviation. Significant *p*-values compared to their corresponding (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$).



Strain	Promoter marked	% GFP fluorescence events (4h)	% GFP fluorescence events (24h)
E. coli ATCC 25922 pMS201:*gfp*mut2	Ø (Negative control)	0.2%	0.1%
	P*cyaA* (Positive control)	92.4%	99.6%
	P*uhpT*	0.6%	0.1%
	P*uhpT* + 25 mg/L G6P	76.9%	7.7%
E. coli MG1655 pMS201:*gfp*mut2	Ø (Negative control)	1.7%	0.7%
	P*cyaA* (Positive control)	99.8%	99.7%
	P*uhpT*	1.2%	0.7%
	P*uhpT* + 25 mg/L G6P	99.7%	60.6%

Strain	Promoter marked	% GFP fluorescence events (4h)	% GFP fluorescence events (24h)
E. coli ATCC 25922 pMS201:*gfp*mut2	Ø (Negative control)	0.2%	0.1%
	P*glpT*	59.3%	97.3%
	P*glpT* + 0.45 mg/mL GLY	80.7%	99.6%
	P*glpT* + 1.8 mg/mL GLY	62.8%	99.7%
	P*glpT* + 7 mg/mL GLY	27.4%	99.9%
E. coli MG1655 pMS201:*gfp*mut2	Ø (Negative control)	1.7%	0.7%
	P*glpT*	1.1%	54.5%
	P*glpT* + 0.45 mg/mL GLY	40.6%	97.5%
	P*glpT* + 1.8 mg/mL GLY	19%	94.7%
	P*glpT* + 7 mg/mL GLY	16.7%	76.4%

Figure 2. Transcriptional analysis of *glpT, uhpT, and cyaA* expression in *E. coli* ATCC25922 and *E. coli* MG1655 at 4 h and 24 h. GFP fluorescence in *E. coli* cells carrying the transcriptional fusions (P_{glpT}-*gfp*, P_{uhpT}-*gfp*, and P_{cyaA}-*gfp*) and grown in MHB alone or supplemented with glucose-6-phosphate (G6P) or glycerol (GLY). The red and yellow areas identify the regions considered negative and positive for the fluorescence signal, respectively (as assessed with cells carrying the empty pMS201vector or with P_{cya}-*gfp* fusion).

2.2. Susceptibility Testing

The fosfomycin MICs, performed with the reference method, for the isogenic collection are shown in Table 1, and the results of disk diffusion are shown in Figure 3.

Table 1. Fosfomycin MIC (mg/L) results by agar dilution without and with 7 mg/mL of glycerol and 25 mg/L of glucose-6-phosphate (G6P).

		Fosfomycin MIC (mg/L)			
		No Glycerol		**Glycerol 7 mg/L**	
		No G6P	**G6P 25 mg/L**	**No G6P**	**G6P 25 mg/L**
E. coli **ATCC25922**		>64	2	64	2
E. coli **MG1655**	**Wild-type**	64	2	32	2
	Δ*glpT*	>64	2	>64	2
	Δ*uhpT*	>64	>64	>64	>64
	Δ*cyaA*	>64	4	>64	2
	Δ*glpR*	4	1	2	1
	Δ*glpK*	>64	4	32	4
	Δ*glpT-uhpT*	>64	>64	>64	>64

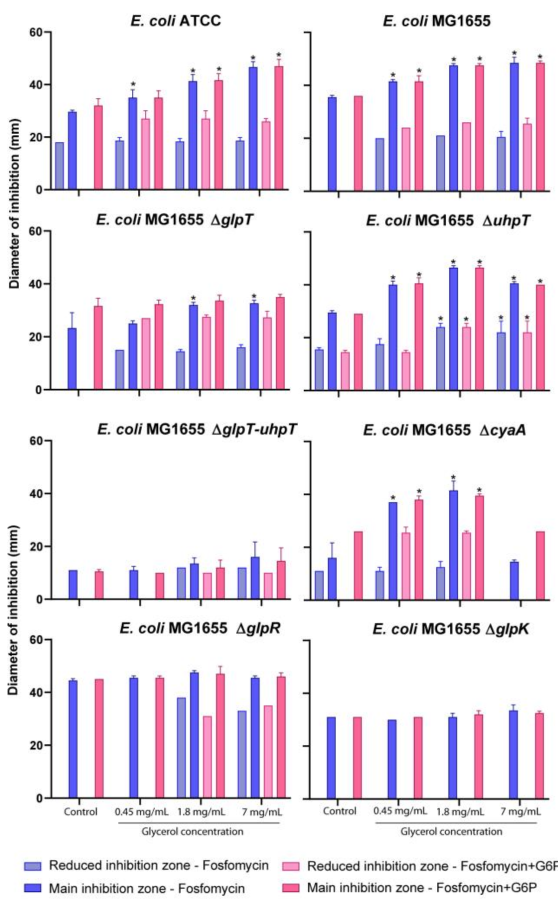


Figure 3. Results of fosfomycin susceptibility by disk diffusion assay with the addition of 0.45, 1.8, and 7 mg/L of glycerol and/or 25 mg/L of glucose-6-phosphate (G6P). The dark-colored columns show the diameter of the main inhibition zone (mean, mm), and the light-colored columns show the diameter of the reduced inhibition zone (mean, mm). Error bars represent the standard deviation. * denotes $p < 0.05$ with respect to their respective control.

With the agar dilution assay, the fosfomycin MIC for *E. coli* ATCC25922 was 2 mg/L with the addition of G6P. The absence of G6P increased the MIC to >64 mg/L, and the addition of 7 mg/mL glycerol did not restore fosfomycin susceptibility. With respect to the *E. coli* MG1655 strain, similar results were observed with a MIC of 2 mg/L with G6P, 64 mg/L without G6P, and 32 mg/L without G6P but with the addition of glycerol. The fosfomycin MIC for the single *uhpT* and the double-gene mutant for both transporters (Δ*glpT-uhpT*) were >64 mg/L for all the assayed conditions. For the remaining mutant strains, the MIC ranged from 1–4 mg/L with the addition of G6P, $\geq$32 mg/L in the absence of G6P, regardless of the addition of glycerol, except the mutant for Δ*glpR* gene (2 and 4 mg/L).

Regarding the results observed by the agar dilution method, the addition of glycerol did not improve fosfomycin activity (MIC drop of $\geq$2 $\log_2$ dilution) with respect to fosfomycin alone or in combination with G6P.

Regarding the disk diffusion assays, considering the main inhibition zone, the results showed that the wild-type strains significantly increased their fosfomycin susceptibility with the addition of glycerol. This increase occurred with and without the addition of G6P for both wild-type strains, except for the *E. coli* ATCC25922 strain with glycerol (0.45 mg/mL) with G6P.

The use of fosfomycin without G6P generated a reduced inhibition zone for the *E. coli* ATCC25922 but not for the *E. coli* MG1655 strains. It should be noted that the addition of glycerol also generated this reduced inhibition zone under certain conditions. The addition of glycerol did not modify the size of the reduced inhibition zone for the *E. coli* ATCC25922 strain but produced a reduced inhibition zone with the addition of G6P, also observed for the *E. coli* MG1655.

With respect to the Δ*glpT* mutant, a significant increase in susceptibility was observed with the addition of 1.8 and 7 mg/mL of glycerol. This increase did not occur with the addition of G6P. Regarding the reduced inhibition zone, no changes were observed with the addition of the different concentrations of glycerol, with or without G6P.

The *E. coli* MG1655 Δ*uhpT* mutant showed, in the reduced inhibition zone, a significant increase in susceptibility with 1.8 and 7 mg/mL of glycerol, with and without G6P. Furthermore, the addition of any concentration of glycerol significantly increased the main inhibition zone, with or without G6P.

E. coli MG1655Δ*cyaA* strain showed a significant increase in susceptibility with 0.45 and 1.8 mg/mL of glycerol. The addition of 7 mg/mL of glycerol did not produce any effect, regardless of the addition of G6P.

E. coli MG1655 Δ*glpT-uhpT,* Δ*glpR,* and Δ*glpK* strains did not show any significant increase in susceptibility in any of the conditions evaluated.

2.3. Fosfomycin and Glycerol Interaction Assay

The result of the interaction assay is shown in Table 2 and Figure S2. The combination of fosfomycin and glycerol showed synergistic activity (ZIP score > +10) G6P for the wild-type strains (*E. coli* ATCC25922: 16.3 $\pm$ 1.2 and *E. coli* MG1655: 16.5 $\pm$ 1.9), and for *E. coli* MG1655 Δ*uhpT* strain with G6P (12.3 $\pm$ 3.6) and without (11.3 $\pm$ 2.9). The most synergistic area occurred between 7–28 mg/mL of glycerol and 0.5–2 mg/L of fosfomycin for the *E. coli* ATCC25922 strain. Similar results were observed for the *E. coli* MG1655 strain, but at 0.25–1 mg/L of fosfomycin. In the case of *E. coli* MG1655 Δ*uhpT*, the greatest synergy was observed at the same concentration as that observed for the *E. coli* ATCC25922 strain with and without G6P.

Table 2. ZIP synergy scores for the combination of fosfomycin with glycerol, with and without glucose-6-phosphate (G6P). Green and red colors show synergistic and antagonistic results, respectively.

ZIP SYNERGY SCORE	ATCC25922	MG1655						
		Wild-Type	Δ*glpT*	Δ*uhpT*	Δ*glpT-uhpT*	Δ*cyaA*	Δ*glpR*	Δ*glpK*
Fosfomycin + Glycerol	16.4 ± 1.2	16.5 ± 1.9	2.5 ± 4.8	11.3 ± 2.9	−12.8 ± 5.4	−0.7 ± 6.4	2.7 ± 1.6	1.7 ± 1.9
Fosfomycin + Glycerol + G6P	6.8 ± 1.9	5.8 ± 2.6	6.9 ± 4.3	12.4 ± 3.6	−21 ± 3.8	1.8 ± 6.6	6.1 ± 1.6	2.7 ± 3.7

Antagonism (ZIP score < −10) was observed against the *E. coli* MG1655 Δ*glpT-uhpT* strain under any condition (−12.8 ± 5.4 and −21 ± 3.8). The addition of glycerol did not interact with the rest of the evaluated strains.

2.4. Fosfomycin and Glycerol Time-Kill Assays

E. coli E. coli ATCC25922, *E. coli* MG1655, and their mutant derivatives were evaluated at two concentrations of fosfomycin (64 and 307 mg/L), without glycerol, and with two concentrations (0.45 and 7 mg/mL). The results are shown in Figure 4 and Figure S3 (for *E. coli* Δ*glpT-uhpT*, Δ*cyaA*, Δ*glpR*, and Δ*glpK strains*).

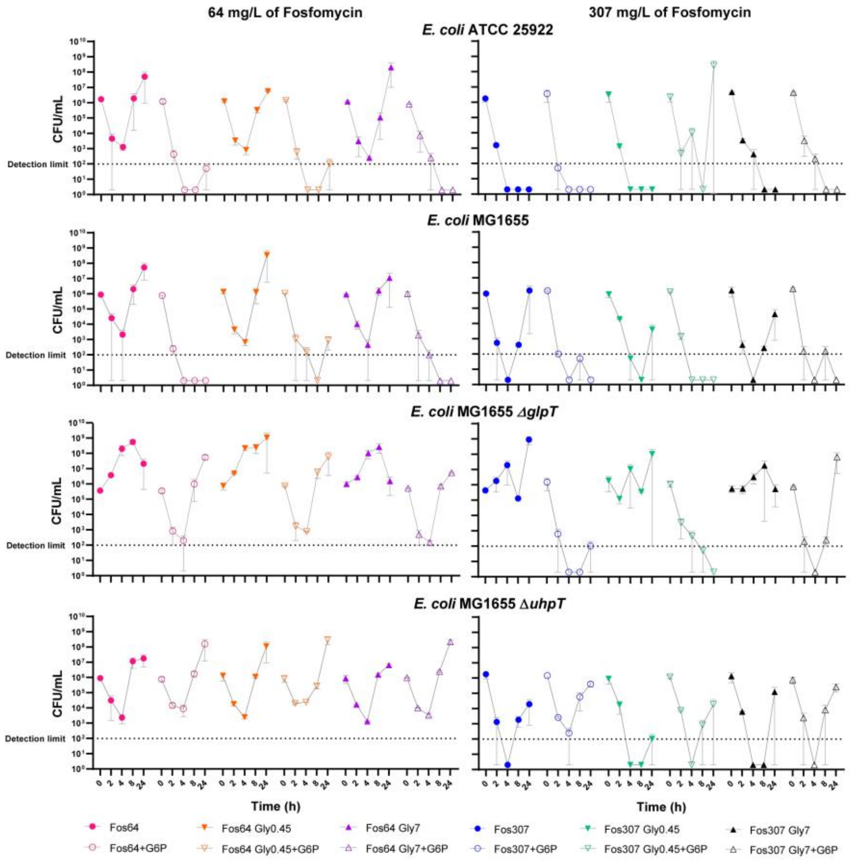


Figure 4. Time-kill assays of fosfomycin alone and in combination with glycerol (0.45 and 7 mg/L) and/or glucose-6-phosphate (G6P, 25 mg/L) against *E. coli* wild-type *E. coli* ATCC25922 and *E. coli* MG1655 wild-type and mutant derivative strains (Δ*glpT* and Δ*uhpT*), at 0, 2, 4, 8, and 24 h. Bacterial concentrations (CFU/mL) are represented as symbols for mean and range.

The addition of glycerol and G6P did not modify growth under control conditions (without fosfomycin, data not shown). Fosfomycin alone at 64 mg/L showed a bactericidal

effect (decreased the bacterial burden $\geq$3 $\log_{10}$ CFU/mL) within the first 4 h against the wild-type and the *E. coli* MG1655 Δ*uhpT* mutant strains, but bacterial regrowths were observed at 8 and 24 h. The addition of G6P also showed a bactericidal effect within the first 4–8 h in all strains except *E. coli* MG1655 Δ*uhpT* and Δ*glpT-uhpT* and prevented the bacterial regrowth for the wild-type strains and the *E. coli* MG1655 Δ*glpR* and Δ*glpK* mutant strains. The addition of glycerol at both tested concentrations did not improve the bactericidal effect against any of the evaluated strains.

Except for *E. coli* MG1655 Δ*glpT* and Δ*glpT-uhpT* strains, fosfomycin alone at 307 mg/L showed a bactericidal effect against all the strains within the first hours. However, bacterial regrowth was observed except for the wild-type *E. coli* ATCC and MG1655 Δ*glpR* strains. The addition of G6P improved the bactericidal effect and prevented the regrowth in all the strains except for *E. coli* MG1655 Δ*uhpT* and Δ*glpT-uhpT.* The addition of glycerol at both concentrations did not improve the bactericidal effect in any of the evaluated strains. Only against *E. coli* MG1655 Δ*uhpT* strain fosfomycin combined with 0.45 mg/mL of glycerol showed an initial improved activity; however, bacterial regrowth was observed after 24 h.

3. Discussion

The present study evaluates the role of glycerol in enhancing fosfomycin activity against wild-type strains of *E. coli* and strains that harbor specific determinants of resistance to fosfomycin.

In general, the activation of the GlpT transporter using glycerol as an internal activator showed contradictory results on the activity of fosfomycin.

The present study shows that glycerol, at physiological concentrations, can activate the *glpT* promoter, increasing *glpT* expression [13,16]. This result agrees with previous works showing the activation of genes (*glpACB*, *glpD glpFKX*, *glpTQ*, etc.) involved in the glycerol catabolism and regulated by the *glpR* repressor in *E. coli*, *P. aeruginosa* or *P. putida* [14,15,17]. However, this glycerol induction of *glpT* shows a delayed initiation in contrast to the rapid activation of the hexoses-6-phosphate transporter (*uhpT*) with the addition of G6P. These effects have been observed using GFP promoter fusions in real-time fluorescence monitoring and flow cytometry assays for *glpT* and *uhpT* genes.

A possible explanation for this behavior was given by Nikel et al. [14], who observed a protracted lag phase in cultures of *P. putida* KT2440 growing in glycerol.

The regulatory network of the *glp* genes needs the product of the first biochemical reaction (sn-glycerol-3-P) to derepress gene expression, which is otherwise inhibited by GlpR. However, the genes that encode the glycerol transporter and the kinase that produces G3P from glycerol are repressed by the GlpR protein. Thus, to get the transcription started is the low-probability effector-independent stochastic lifting of the repression. While the derepression process is taking place, this transcriptional architecture translates into different levels of metabolic activity (representing, in this context, the ability of the cells to catabolize glycerol). It is important to note that while the *glpT* gene is absent in *P. putida,* the regulation network is conserved in *E. coli* and *P. aeruginosa*. [1,15,18]

With regard to fosfomycin activity with the addition of glycerol, the present study shows distinct results depending on the characteristics of the assay.

In the disk diffusion assays, secondary and reduced inhibition zones were observed, suggesting the presence of subpopulations with different fosfomycin susceptibility, even with the use of glycerol and G6P. The maximum fosfomycin activity was observed against the *glpR* repressor mutant because the *glpT* transporter is fully derepressed and insensitive to the addition of glycerol. Additionally, the addition of glycerol did not show any effect in *glpK* mutant due to the inability to transform glycerol into glycerol-3-phosphate [16].

However, fosfomycin activity was observed, similar to that observed against the *E. coli* MG1655 wild-type strain without glycerol, and this could be partially explained by the intracellular biosynthesis of sn-glycerol-3-phosphate as a precursor of phospholipid synthesis [19]. The absence of activity was observed in the double-gen mutant Δ*glpT-uhpT*

strain, indicating that the activation of the fosfomycin transporters is the main control factor of fosfomycin activity, as previously observed by Ballesteros et al. [1].

It is also important to note that the reduced inhibition zones remained unchanged, irrespective of the glycerol concentration or the addition of G6P, suggesting the presence of baseline defects in the complex regulatory networks of the fosfomycin transporters *glpT* and *uhpT* [6,20].

In the present study, discrepancies between disk diffusion and agar dilution susceptibility assays have been observed. These results agree with our previous studies in which fosfomycin susceptibility showed inconsistent results between broth and agar dilutions and agar diffusion techniques in collections of clinical isolates and isogenic mutants related to fosfomycin resistance, including heteroresistant strains [1,21,22]. Although there were no differences in the MIC of fosfomycin when glycerol was added to the assay, it must be noted that lower bacterial densities were observed (data not shown), indicating a mild synergistic effect not measurable with the assay. Regarding the interaction assays between fosfomycin and glycerol, only the wild-type strains and *E. coli* MG1655 Δ*uhpT* mutant showed synergistic activity. However, the time-kill assays did not show sensitization with the combination of fosfomycin and glycerol. It is important to note that in the time-kill assays, divergent results were observed between the replicates for wild-type strains and for the *E. coli* MG1655 Δ*uhpT*. For these strains, one replicate of the time-kill assay showed a total bacterial clearance and the other a bacterial regrowth after 24 h, which partially agrees with the results observed in the interaction assay. This divergence found between these replicates could be explained considering the rapid bactericidal activity of fosfomycin in contrast to our previous results showing the delayed activation of the *glpT* transporter, increasing the probability of the emergence of fosfomycin-resistant subpopulations as commonly observed in previous studies [1,23].

In conclusion, glycerol showed increased expression of the fosfomycin transporter *glpT* and a synergistic effect with fosfomycin in the interaction and disk diffusion assays. However, this molecule does not appear to be a good candidate as an adjuvant to fosfomycin therapy since the delay in the induction of *glpT* allows the selection of resistant subpopulations. It would therefore be necessary to perform further in vitro and in vivo studies aimed at overcoming these problems.

4. Materials and Methods

4.1. Bacterial Strains

E. coli ATCC25922 and *E. coli* MG1655 were used as the reference strain, and six isogenic mutants from *E. coli* MG1655 mutants (Δ*glpT*, Δ*uhpT*, Δ*glpT-uhpT*, Δ*cyaA*, Δ*glpR*, and Δ*glpK*) were used in the assays. Isogenic mutants were generated from the KEIO collection [24] using phage P1*vir* transduction (Coli Genetic Stock Center [CGSC], Yale University, New Haven, CT, USA) as previously described [25].

4.2. Activation Kinetic of Fosfomycin Transporters Promoters

The activity of the fosfomycin transporter promoters *glpT* and *uhpT* was evaluated by monitoring the fluorescence accumulation in *E. coli* ATCC25922 and *E. coli* MG1655 carrying pMS201-P*glpT*::*gfp*mut2 or pMS201-P*uhpT*::*gfp*mut2 reporters, as described by Zaslaver et al. [26]. Bacterial culture fluorescence was determined after transferring overnight cultures in MHB to 96-well plates (Nunclon Delta Surface, Thermo Scientific, Waltham, MA, USA) with 200µL of MHB per well. Starting bacterial concentrations were adjusted to 5×10^5 CFU/mL. The assays were carried out in Mueller Hinton Broth II (MHB) alone or supplemented with 0.45 mg/mL, 1.8 mg/mL, and 7 mg/mL of glycerol with and without G6P (Sigma-Aldrich, Madrid, Spain). Low, medium, and high glycerol physiological concentrations were assayed in combination with fosfomycin. The low glycerol concentration of 0.45 mg/mL corresponded to a target effective concentration to reduce the intracranial pressure [12]. The high glycerol concentration of 7 mg/mL corresponded to steady-state serum concentrations in patients with normal hepatic and renal function following constant

intravenous infusion of 0.87 g/kg/h [27]. Finally, the medium glycerol concentration of 1.8 mg/mL was selected, corresponding to an intermediate concentration between 0.45 and 7 mg/mL in a $\log_2$ dilution scale. Green fluorescence (excited at 485 nm and measured at 540 nm) and bacterial growth (measured at 595 nm) were monitored each hour for 24 h with an Infinite200 Pro plate reader (Tecan Group AG, Männedorf, Switzerland). The assays were performed in duplicate. The OD:fluorescence ratio of the promoterless construction (pMS201-Ø::*gfp*mut2) was used as background for all experiments under the different growth conditions. Fluorescence was normalized to the OD, and the background was subtracted. The results were compared with ANOVA and Tukey's multiple comparison test.

4.3. Population Analysis of Fosfomycin Transporters Promoters Induction

Flow cytometry assays were conducted to assess the population distribution of *glpT* and *uhpT* expressions (pMS201-P*glpT*::*gfp*mut2 or pMS201-P*uhpT*::*gfp*mut2 reporters) in *E. coli* ATCC25922 and *E. coli* MG1655. Furthermore, a negative expression control (pMS201-Ø::*gfp*mut2) and positive expression control (pMS201-P*cyaA*::*gfp*mut2) were used as described. Overnight cultures were diluted in 20 mL adjusting a bacterial concentration of 5×10^5 CFU/mL. Bacterial growths were carried out in MHB, MHB supplemented with 25 mg/L of G6P, and MHB supplemented with glycerol: 0.45 mg/mL, 1.8 mg/mL, and 7 mg/mL. A milliliter sample of each condition was taken, and fluorescence was monitored at 4 and 24 h. The Beckman Coulter FC500 cytometer (Beckman Coulter, United States) was used for size (FSC) and complexity (SSC) measurements to define the bacterial population. Green fluorescence was excited using a blue laser (488 nm) and measured at 530/30 nm. The promoterless construction (pMS201-Ø::*gfp*mut2) was used as a negative control, and pMS201-P*cyaA*::*gfp*mut2 was used as a positive control.

4.4. Susceptibility Testing

Fosfomycin MIC was performed using the reference method, the agar dilution assay, following EUCAST standards [28]. Mueller Hinton II agar (MHA) plates (Sigma-Aldrich, Madrid, Spain), with and without 25 mg/L of G6P, with and without 7 mg/mL of glycerol. The fosfomycin (Sigma-Aldrich) concentration ranged from 0.5 to 64 mg/L. Plates were dried and incubated for 20 h at 35 °C. The assays were performed in duplicate.

Fosfomycin susceptibility was also determined using the disk diffusion method using blank antimicrobial disks loaded with 200µg of fosfomycin alone or with 50µg of G6P, following EUCAST recommendations [28]. Additionally, fosfomycin activity was assayed by supplementing MHA plates with the addition of 0.45 mg/mL, 1.8 mg/mL, and 7 mg/mL of glycerol. The diameter of the main and any other reduced inhibition zone was measured. The addition of glycerol was compared with respect to the negative control (MHA with or without G6P). The results were compared with ANOVA and Tukey's multiple comparison test. The assays were performed in triplicate.

4.5. Fosfomycin and Glycerol Interaction Assay

The interaction between fosfomycin and glycerol was studied using the checkerboard assay in duplicate. Briefly, the interaction assay was performed with an inoculum of 5×10^5 CFU/mL in 96-well plates with a final volume of 200 µL per well. Assays were performed with MHB with and without 25 mg/L G6P. Fosfomycin concentrations ranged from 0.125 to 128 mg/L, and glycerol concentrations from 0.45 to28.8 mg/mL. Wells without fosfomycin or glycerol were used as single-drug assays or growth controls. Bacterial densities were quantified spectrophotometrically by measuring optical density at 595 nm using an Infinite200 Pro plate reader. Bacterial viability was calculated as the ratio of the final bacterial OD to the final bacterial OD of the control growth well (without drug). A four-parameter log-logistic model was fitted to the data to generate dose–response curves for fosfomycin and glycerol. The degree of drug synergy across the entire dose–response matrix was analyzed using the response surface model, zero interaction potential

(ZIP) [29]. The ZIP model assumes that two noninteracting drugs are expected to incur minimal changes in their dose–response curves. A delta score was calculated to quantify the deviation from the expectation of ZIP for a given dose pair and used the average delta over a dose–response matrix as a summary interaction score for a drug combination. Model construction and synergy studies were performed with the Synergyfinder package for R [29]. A synergy score of <-10 was considered antagonistic, a range from -10 to 10 as additive, and >10 as synergistic.

4.6. Fosfomycin and Glycerol Time-Kill Assays

Time-kill assays were performed in duplicate using fosfomycin concentrations of 0 (as growth control), 64, and 307 mg/L with and without 25 mg/L G6P, with and without 0.45 and 7 mg/mL of glycerol. The activity of fosfomycin alone at concentrations of 64 mg/L (lowest concentrations of fosfomycin in the non-susceptible category according to EUCAST breakpoints) and at 307 mg/L (mean maximum plasma concentrations in humans at steady-state after a dose of fosfomycin 8 g/Q8h), respectively, was determined [28,30].

Briefly, isolated colonies overnight of each strain were used to prepare the pre-inoculum in MHB and incubated overnight with shaking at 37 °C. The starting inoculum was set at 5×10^5 CFU/mL in a final volume of 20 mL, and bacterial cultures were incubated at 37 °C with shaking. The number of viable CFUs was determined at 0, 2, 4, 8, and 24 h by serial dilution, followed by plating on MH agar plates with or without 64 mg/L fosfomycin and 25 mg/L G6P. The number of colonies was counted after 24 h of incubation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antibiotics11111612/s1, Figure S1: Assay of promoter activities in response to glycerol (GLY) and glucose-6-phosphate (G6P). Time-course quantification of GFP expression as a measure of induction of the *glpT* and *uhpT* genes in the ATCC25922 and MG1655 strains after 4, 12, and 24 h of exposure. The data were normalized to promoterless strains. Error bars represent standard deviations. Concentrations of glycerol (Gly) of 0.45, 1.8, and 7 mg/mL and glucose-6-phosphate (G6P) of 25 mg/L were used as inductors; Figure S2: Interaction assay of fosfomycin in combination with glycerol against *Escherichia coli* ATCC25922 and MG1655 strains, represented as heat maps. The red and green areas represent synergy and antagonism, respectively. The white rectangles show the maximum synergistic area. The concentration–response curves for fosfomycin and glycerol alone are found on the left side of each heatmap. Figure S3: Time-kill assays of fosfomycin alone and in combination with glycerol (0.45 and 7 mg/L) and/or glucose-6-phosphate (G6P, 25 mg/L) against *E. coli* wild-type *E. coli* MG1655 mutant derivative strains (Δ*glpT-uhpT*, Δ*cyaA*, Δ*glpR*, and Δ*glpK*), at 0, 2, 4, 8, and 24 h. Bacterial concentrations (CFU/mL) are represented as symbols for mean and range.

Author Contributions: Conceptualization: M.O.-P., I.P.-C. and F.D.-P.; methodology: M.O.-P., J.M.R.-M. and F.D.-P.; validation: M.O.-P., I.P.-C. and F.D.-P.; formal analysis: M.O.-P. and F.D.-P.; investigation: M.O.-P. and A.V.-E.; resources: Á.P., J.M.R.-M. and F.D.-P.; writing-original draft preparation: M.O.-P. and F.D.-P.; writing—review and editing: M.O.-P., I.P.-C., J.R.-B. and F.D.-P.; visualization: M.O.-P. and F.D.-P.; supervision: Á.P., J.M.R.-M. and F.D.-P.; project administration: J.M.R.-M. and F.D.-P.; funding acquisition: J.R.-B., Á.P. and F.D.-P. All authors have read and agreed to the published version of the manuscript.

Funding: This study has been funded by Instituto de Salud Carlos III through the projects PI19/01645 and REIPI RD16/0016/0001 (co-funded by European Regional Development Fund "Away to Make Europe"). Miriam Ortiz-Padilla is supported by a PFIS grant from the Instituto de Salud Carlos III (FI17/00235). Inés Portillo-Calderón is supported by a Rio Hortega grant from the Instituto de Salud Carlos III (CM20/0092). (Co-funded by European Social Fund "Investing in your future"). The funders had no role in the design, collection of data, analysis, and writing of the manuscript or the decision to publish.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Acknowledgments: This study was presented in part at ECCMID 2022, Lisbon, Portugal (Poster Presentation P0794).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Ballestero-Téllez, M.; Docobo-Pérez, F.; Portillo-Calderón, I.; Rodríguez-Martínez, J.M.; Racero, L.; Ramos-Guelfo, M.S.; Blázquez, J.; Rodríguez-Baño, J.; Pascual, A. Molecular insights into fosfomycin resistance in *Escherichia coli*. *J. Antimicrob. Chemother.* **2017**, *72*, 1303–1309. [CrossRef]
2. Prestinaci, F.; Pezzotti, P.; Pantosti, A. Antimicrobial resistance: A global multifaceted phenomenon. *Pathog Glob Health* **2015**, *109*, 309–318. [CrossRef] [PubMed]
3. Cassini, A.; Högberg, L.D.; Plachouras, D.; Quattrocchi, A.; Hoxha, A.; Simonsen, G.S.; Colomb-Cotinat, M.; Kretzschmar, M.E.; Devleesschauwer, B.; Cecchini, M.; et al. Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: A population-level modelling analysis. *Lancet Infect. Dis.* **2019**, *19*, 56–66. [CrossRef]
4. Docobo-Pérez, F.; Drusano, G.L.; Johnson, A.; Goodwin, J.; Whalley, S.; Ramos-Martín, V.; Ballestero-Tellez, M.; Rodriguez-Martinez, J.M.; Conejo, M.C.; van Guilder, M.; et al. Pharmacodynamics of fosfomycin: Insights into clinical use for antimicrobial resistance. *Antimicrob. Agents Chemother.* **2015**, *59*, 5602–5610. [CrossRef] [PubMed]
5. Falagas, M.E.; Maraki, S.; Karageorgopoulos, D.E.; Kastoris, A.C.; Mavromanolakis, E.; Samonis, G. Antimicrobial susceptibility of multidrug-resistant (MDR) and extensively drug-resistant (XDR) Enterobacteriaceae isolates to fosfomycin. *Int. J. Antimicrob. Agents* **2010**, *35*, 240–243. [CrossRef]
6. Marger, M.D.; Saier, M.H.J. A major superfamily of transmembrane facilitators that catalyse uniport, symport and antiport. *Trends Biochem. Sci.* **1993**, *18*, 13–20. [CrossRef]
7. Franchini, A.G.; Ihssen, J.; Egli, T. Effect of Global Regulators RpoS and Cyclic-AMP/CRP on the Catabolome and Transcriptome of *Escherichia coli* K12 during Carbon- and Energy-Limited Growth. *PLoS ONE* **2015**, *10*, e0133793. [CrossRef]
8. Sakamoto, Y.; Furukawa, S.; Ogihara, H.; Yamasaki, M. Fosmidomycin resistance in adenylate cyclase deficient (cya) mutants of *Escherichia coli*. *Biosci. Biotechnol. Biochem.* **2003**, *67*, 2030–2033. [CrossRef]
9. Kadner, R.J.; Shattuck-Eidens, D.M. Genetic control of the hexose phosphate transport system of *Escherichia coli*: Mapping of deletion and insertion mutations in the uhp region. *J. Bacteriol.* **1983**, *155*, 1052–1061. [CrossRef] [PubMed]
10. Yang, B.; Gerhardt, S.G.; Larson, T.J. Action at a distance for glp repressor control of glpTQ transcription in *Escherichia coli* K-12. *Mol. Microbiol.* **1997**, *24*, 511–521. [CrossRef]
11. Santoro, A.; Cappello, A.R.; Madeo, M.; Martello, E.; Iacopetta, D.; Dolce, V. Interaction of fosfomycin with the Glycerol 3-phosphate Transporter of *Escherichia coli*. *Biochim. Biophys. Acta (BBA)-Gen. Subj.* **2011**, *1810*, 1323–1329. [CrossRef] [PubMed]
12. Frank, M.S.; Nahata, M.C.; Hilty, M.D. Glycerol: A review of its pharmacology, pharmacokinetics, adverse reactions, and clinical use. *Pharmacotherapy* **1981**, *1*, 147–160. [CrossRef] [PubMed]
13. Lin, E.C. Glycerol dissimilation and its regulation in bacteria. *Annu. Rev. Microbiol.* **1976**, *30*, 535–578. [CrossRef]
14. Nikel, P.I.; Romero-Campero, F.J.; Zeidman, J.A.; Goñi-Moreno, Á.; de Lorenzo, V. The glycerol-dependent metabolic persistence of Pseudomonas putida KT2440 reflects the regulatory logic of the GlpR repressor. *MBio* **2015**, *6*, e00340-15. [CrossRef] [PubMed]
15. Koch, J.P.; Hayashi, S.; Lin, E.C. The Control of Dissimilation of Glycerol and L-α-Glycerophosphate in *Escherichia coli*. *J. Biol. Chem.* **1964**, *239*, 3106–3108. [CrossRef]
16. Martínez-Gómez, K.; Flores, N.; Castañeda, H.M.; Martínez-Batallar, G.; Hernández-Chávez, G.; Ramírez, O.T.; Gosset, G.; Encarnación, S.; Bolivar, F. New insights into Escherichia coli metabolism: Carbon scavenging, acetate metabolism and carbon recycling responses during growth on glycerol. *Microb. Cell Factories* **2012**, *11*, 46. [CrossRef]
17. Nikel, P.I.; Kim, J.; de Lorenzo, V. Metabolic and regulatory rearrangements underlying glycerol metabolism in Pseudomonas putidaKT2440. *Environ. Microbiol.* **2014**, *16*, 239–254. [CrossRef]
18. Castañeda-García, A.; Rodríguez-Rojas, A.; Guelfo, J.R.; Blázquez, J. The Glycerol-3-Phosphate Permease GlpT Is the Only Fosfomycin Transporter in Pseudomonas aeruginosa. *J. Bacteriol.* **2009**, *191*, 6968–6974. [CrossRef]
19. Edgar, J.R.; Bell, R.M. Biosynthesis in *Escherichia coli* of sn-glycerol-3-phosphate, a precursor of phospholipid. Further kinetic characterization of wild type and feedback-resistant forms of the biosynthetic sn-glycerol-3-phosphate dehydrogenase. *J. Biol. Chem.* **1980**, *255*, 3492–3497. [CrossRef]
20. Castañeda-García, A.; Blázquez, J.; Rodríguez-Rojas, A. Molecular mechanisms and clinical impact of acquired and intrinsic fosfomycin resistance. *Antibiotics* **2013**, *2*, 217–236. [CrossRef]
21. Ballestero-Téllez, M.; Docobo-Pérez, F.; Rodríguez-Martínez, J.; Conejo, M.; Ramos-Guelfo, M.; Blázquez, J.; Rodríguez-Baño, J.; Pascual, A. Role of inoculum and mutant frequency on fosfomycin MIC discrepancies by agar dilution and broth microdilution methods in Enterobacteriaceae. *Clin. Microbiol. Infect.* **2017**, *23*, 325–331. [CrossRef]

22. Portillo-Calderón, I.; Ortiz-Padilla, M.; Rodríguez-Martínez, J.M.; De Gregorio-Iaria, B.; Blázquez, J.; Rodríguez-Baño, J.; Pascual, A.; Docobo-Pérez, F. Contribution of hypermutation to fosfomycin heteroresistance in *Escherichia coli*. *J. Antimicrob. Chemother.* **2020**, *75*, 2066–2075. [CrossRef]
23. Portillo-Calderón, I.; Ortiz-Padilla, M.; de Gregorio-Iaria, B.; Merino-Bohorquez, V.; Blázquez, J.; Rodríguez-Baño, J.; Rodríguez-Martínez, J.M.; Pascual, A.; Docobo-Pérez, F. Activity of fosfomycin and amikacin against fosfomycin-heteroresistant *Escherichia coli* strains in a hollow-fiber infection model. *Antimicrob. Agents Chemother.* **2021**, *65*, e02213-20. [CrossRef]
24. Baba, T.; Ara, T.; Hasegawa, M.; Takai, Y.; Okumura, Y.; Baba, M.; Datsenko, K.A.; Tomita, M.; Wanner, B.L.; Mori, H. Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: The Keio collection. *Mol. Syst. Biol.* **2006**, *2*, 2006.0008. [CrossRef] [PubMed]
25. Thomason, L.C.; Costantino, N.; Court, D.L. *E. coli* genome manipulation by P1 transduction. *Curr. Protoc. Mol. Biol.* **2007**, *79*, 1.17.1–1.17.8. [CrossRef]
26. Zaslaver, A.; Bren, A.; Ronen, M.; Itzkovitz, S.; Kikoin, I.; Shavit, S.; Liebermeister, W.; Surette, M.G.; Alon, U. A comprehensive library of fluorescent transcriptional reporters for *Escherichia coli*. *Nat. Methods* **2006**, *3*, 623–628. [CrossRef] [PubMed]
27. Snyder, J.; Powner, D.; Grenvik, A. Neurologic intensive care. In *Anesthesia and Neurosurgery*; Cottrell, J., Turndorf, H., Eds.; Mosby: Maryland Heights, MI, USA, 1980; pp. 322–360.
28. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint Tables for Interpretation of MICs and Zone Diameters. Version 12.0. 2022. Available online: http://www.eucast.org (accessed on 21 May 2022).
29. Yadav, B.; Wennerberg, K.; Aittokallio, T.; Tang, J. Searching for Drug Synergy in Complex Dose–Response Landscapes Using an Interaction Potency Model. *Comput. Struct. Biotechnol. J.* **2015**, *13*, 504–513. [CrossRef] [PubMed]
30. Pfausler, B. Concentrations of fosfomycin in the cerebrospinal fluid of neurointensive care patients with ventriculostomy-associated ventriculitis. *J. Antimicrob. Chemother.* **2004**, *53*, 848–852. [CrossRef] [PubMed]

Supplementary Material of Effect of Glycerol on Fosfomycin Activity *against Escherichia coli.*

Figure S1. Assay of promoter activities in response to glycerol (GLY) and glucose-6-phosphate (G6P). Time-course quantification of GFP expression as a measure of induction of the glpT and uhpT genes in the ATCC25922 and MG1655 strains after 4, 12, and 24 h of exposure. The data were normalized to promoterless strains. Error bars represent standard deviations. Concentrations of glycerol (Gly) of 0.45, 1.8, and 7 mg/mL and glucose-6-phosphate (G6P) of 25 mg/L were used as inductors.

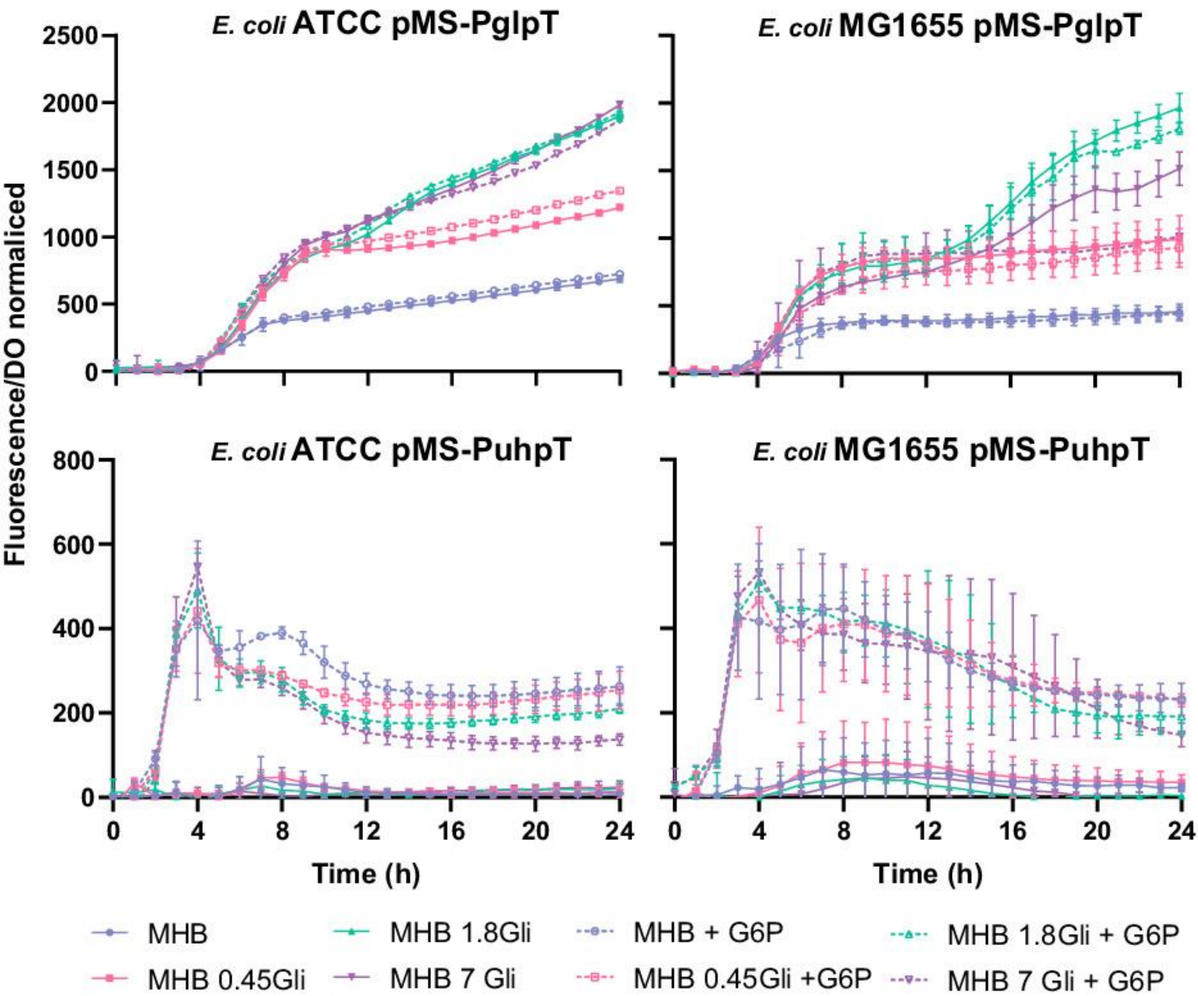


Figure S2. Interaction assay of fosfomycin in combination with glycerol against *Escherichia coli* ATCC25922 and MG1655 strains, represented as heat maps. The red and green areas represent synergy and antagonism, respectively. The white rectangles show the maximum synergistic area. The concentration–response curves for fosfomycin and glycerol alone are found on the left side of each heatmap.

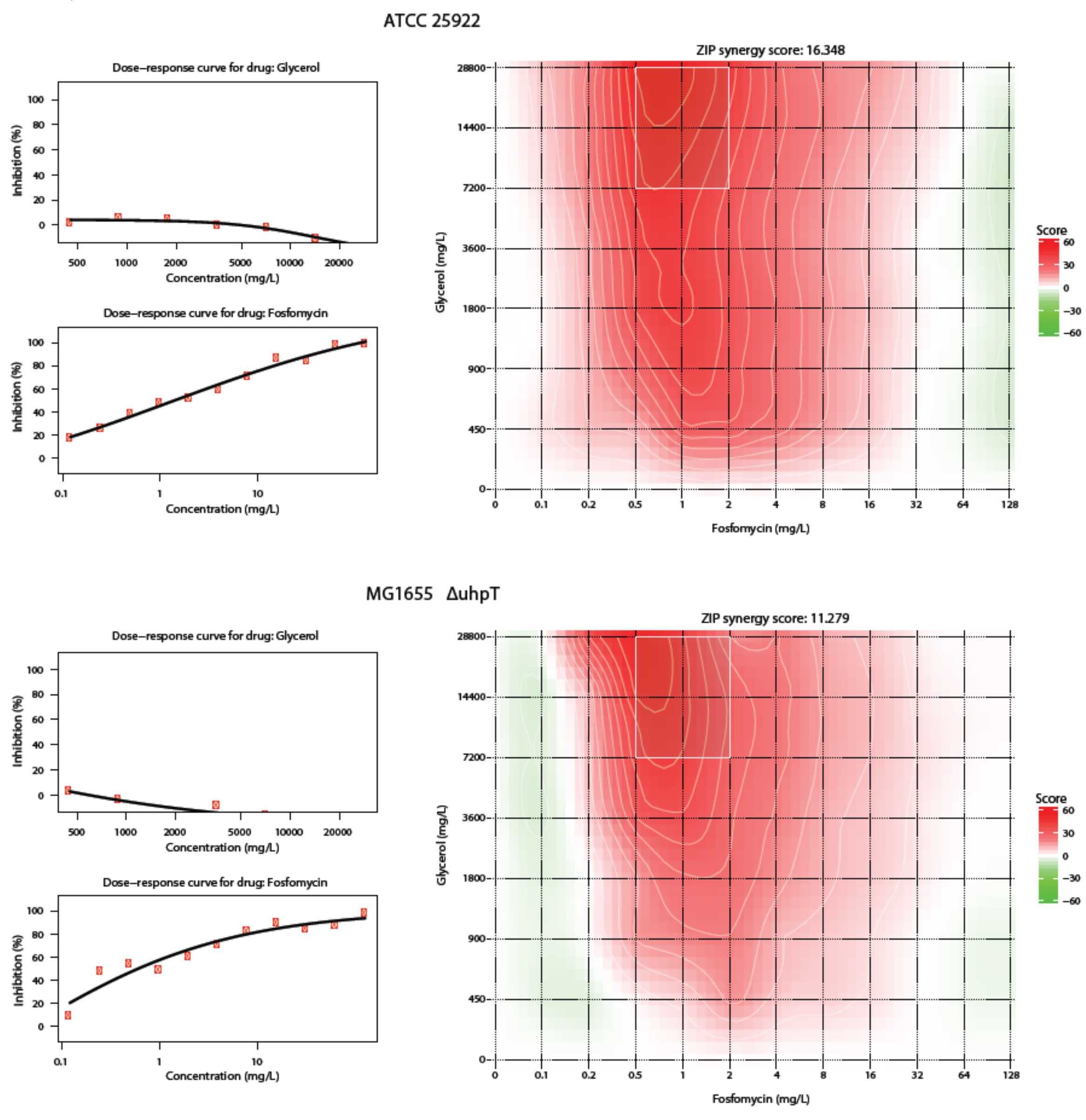


Figure S2. (continued) Interaction assay of fosfomycin in combination with glycerol against *Escherichia coli* ATCC25922 and MG1655 strains, represented as heat maps. The red and green areas represent synergy and antagonism, respectively. The white rectangles show the maximum synergistic area. The concentration–response curves for fosfomycin and glycerol alone are found on the left side of each heatmap.

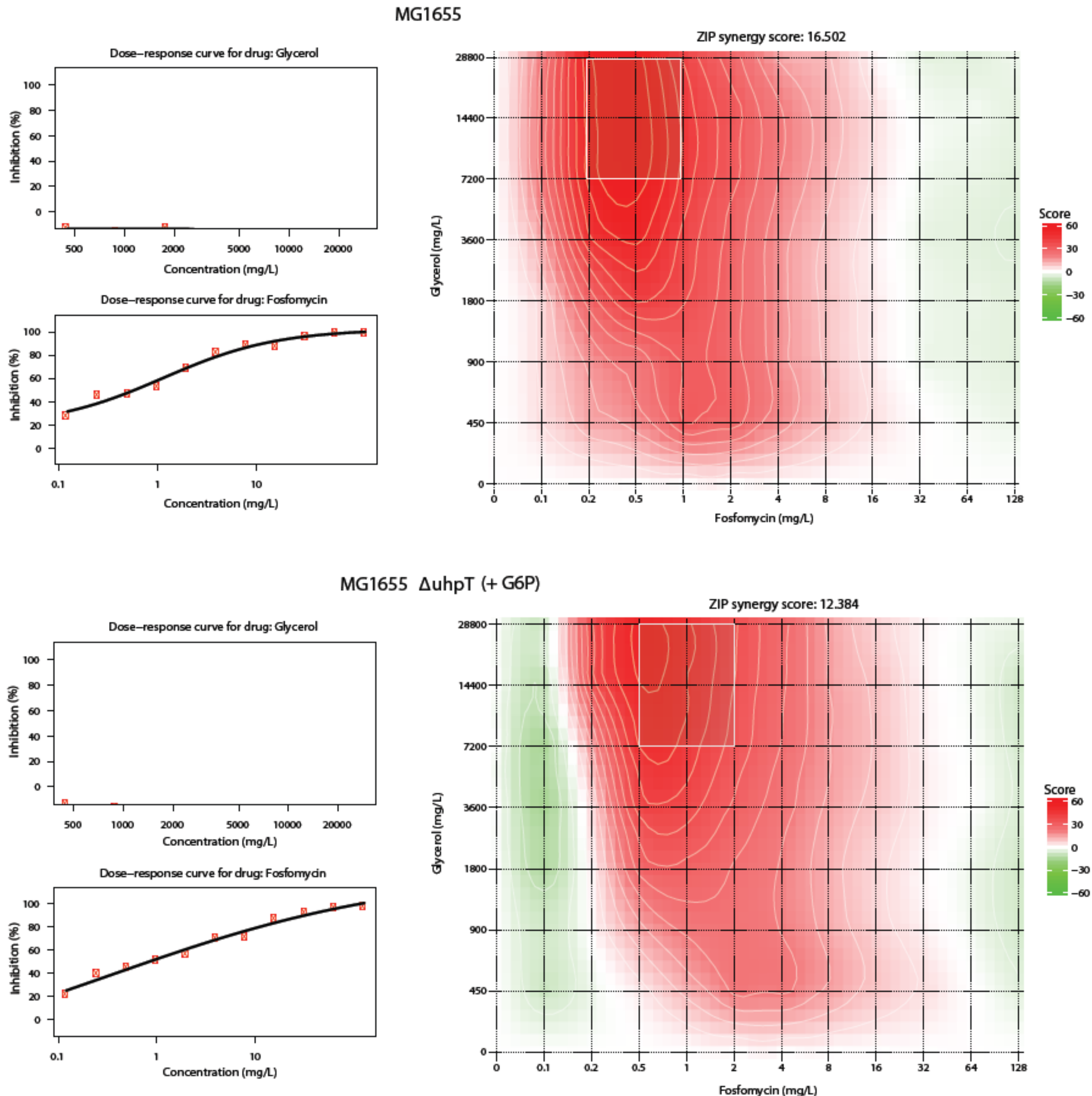
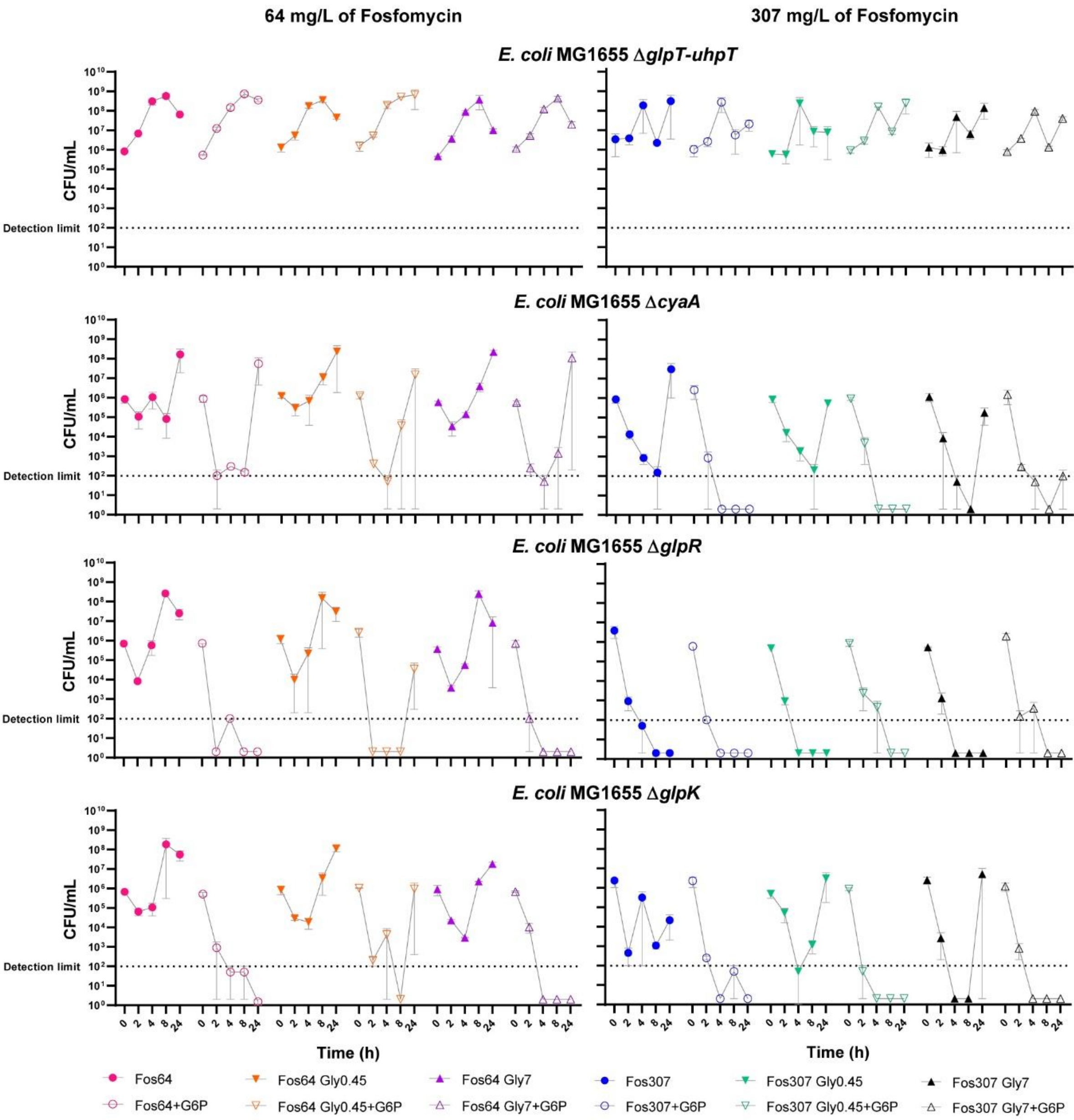


Figure S3. Time-kill assays of fosfomycin alone and in combination with glycerol (0.45 and 7 mg/L) and/or glucose-6-phosphate (G6P, 25 mg/L) against *E. coli* wild-type *E. coli* MG1655 mutant derivative strains (Δ*glpT-uhpT,* Δ*cyaA,* Δ*glpR,* and Δ*glpK*), at 0, 2, 4, 8, and 24 h. Bacterial concentrations (CFU/mL) are represented as symbols for mean and range.



3. Role of inorganic phosphate concentrations in in vitro activity of fosfomycin.

The lack of development of new antibiotics, together with the problem of resistance, has made it necessary to revive older antibiotics, as fosfomycin. It's an antimicrobial approved for the treatment of uncomplicated urinary tract infections, soft-tissue infection and sepsis caused by *Enterobacteriaceae.*

Uptake of fosfomycin leads to the simultaneous counterflow of inorganic phosphate (Pi) from the cell via an antiport mechanism, the hexose phosphate (UhpT) and glycerol-3-phosphate (GlpT) membrane transporters. The objective of this study was to evaluate the in vitro activity of fosfomycin under different physiological concentrations of Pi.

The results obtained in this work are the following:

Whole-genome sequencing

Analyses of translated nucleotide sequences of genes associated with fosfomycin resistance or regulation (Table S1, Art. 3) showed no mutations in Crp and UhpA and no deletions or insertions were detected in the rest of the amino acid sequences associated with fosfomycin resistance, except for the Ec20190430 isolate, which showed deletions in GlpT and UhpB, and Ec20190178, which presented a deletion causing a frameshift mutation and a premature stop codon. With respect to proteins involved in the bacterial phosphate regulatory system (Table S2, Art. 3), only PitA and PhoB showed WT sequences. The rest of the amino acid sequences likewise showed many polymorphisms. Nevertheless, the biological significance of many of the observed polymorphisms is unclear.

Susceptibility testing

The fosfomycin MICs for the isogenic collection and clinical isolates are shown in Table 1 (Art.3). The isogenic collection and clinical isolates showed a decrease in fosfomycin activity linked to increased concentrations of Pi. When the Pi concentration in MHA increased from 1 to 13mM, 1-4-log_2 dilution differences were observed. Clinical category changes were observed for Δ*glpT* and Ec20190736, Ec20200150, and Ec20200178. An increase to 42mM showed 1-8 log_2 dilution differences, and all the susceptible strains changed to resistant. The Δ*uhpT* and Δ*glpT-uhpT* mutants, Ec20190430 (Δ*glpT-uhpB)* and Ec20190556 were resistant irrespective of the Pi concentration. The isogenic mutant Δ*phoB* and the wild-type strain showed similar fosfomycin MICs compared with wild-type strain at all Pi concentrations tested.

Fosfomycin transport induction

Peak fosfomycin transporter promoter activities (at 4h), evaluated at Pi concentrations of 1, 13 and 42mM ±25mg/L of glucose-6-phosphate, are shown in Figure S1 (Art. 3). The increased Pi

concentration did not cause significant changes in *glpT* or *uhpT* transcription. Only the addition of glucose-6-phosphate significantly increased *uhpT* transcription.

Fosfomycin activity assay

Fosfomycin EC_{50} results are shown in Table 2 (Art. 3). Figure S2 (Art. 3) shows the 24h growth monitoring assays in the presence of fosfomycin under increasing concentrations of Pi.

By increasing the Pi concentrations in MHB, significantly higher viability and fosfomycin EC_{50} were observed, except for the double mutant Δ*glpT-uhpT.*

Increasing the Pi concentration to 13mM shifted viability curves slightly to the right and raised the fosfomycin EC_{50} for the wild-type and Δ*glpT* with respect to 1mM Pi. Concentrations of 42 mM Pi also increased bacterial viability at higher fosfomycin concentrations and showed a significant increase in fosfomycin EC_{50} with respect to 1 and 13mM Pi in the wild-type, Δ*glpT* and Δ*uhpT*.

When Pi was increased from 1 to 13 mM, there was a 2-log_2 increase in fosfomycin concentration able to inhibit bacterial viability, while an increase to 42 mM Pi led to a 4-log_2 dilution increase in the wild-type and Δ*uhpT* strains, and an 8-log_2 dilution increase in the Δ*glpT* strain. None of these changes however were observed for the double mutant Δ*glpT-uhpT* as the Pi concentration increased.

Time-kill assay

The results of the time-kill assays of *E. coli* BW25113 and the isogenic mutants (Δ*glpT*, Δ*uhpT* and Δ*glpT-uhpT*) evaluated at three fosfomycin concentrations (307, 1053 and 4015 mg/L) under two different Pi concentrations (1mM and 28mM) are shown in figure S3 (Art. 3). All the tested strains at the two concentrations of Pi in the absence of fosfomycin showed similar growth. All fosfomycin concentrations showed bactericidal activity (>3 log CFU/mL decrease) against the wild-type strain. It should be noted that 307 mg/L of fosfomycin was unable to clear the bacterial culture at 28 mM Pi. Similar results were observed in the Δ*glpT* strain at the low Pi concentration. At 28 mM Pi, however, bacterial regrowth occurred at fosfomycin concentrations of 307 and 1053 mg/L.

With respect to the Δ*uhpT* strain, the bacterial counts dropped in the first 4 hours at all fosfomycin concentrations, although regrowth was observed at 307 mg/L of fosfomycin after 8h with both Pi concentrations, and at 1053 mg/L of fosfomycin with 28 mM Pi. Finally, against the double mutant (Δ*glpT-uhpT*), not even 4015 mg/L of fosfomycin was able to sterilise the bacterial culture with 28mM Pi after 24h. However, early fosfomycin activity was observed at 1053 and 4015 mg/L with both Pi concentrations, although bacterial regrowth was observed with the lowest fosfomycin concentration and a static effect with the highest.

Clinical Microbiology and Infection 28 (2022) 302.e1–302.e4

Contents lists available at ScienceDirect

Clinical Microbiology and Infection

journal homepage: www.clinicalmicrobiologyandinfection.com

Research note

Role of inorganic phosphate concentrations in *in vitro* activity of fosfomycin

Miriam Ortiz-Padilla [1, 2, *], Inés Portillo-Calderón [1, 2, *], Natalia Maldonado [1, 2], José Rodríguez-Martínez [2, 3], Belén de Gregorio-Iaria [1], Vicente Merino-Bohórquez [4, 5], Jesús Rodríguez-Baño [1, 2, 6], Álvaro Pascual [1, 2, 3, †], Fernando Docobo-Pérez [2, 3, *, †]

[1] *Unidad de Gestión Clínica de Enfermedades Infecciosas y Microbiología Clínica, Hospital Universitario Virgen Macarena, Seville, Spain*
[2] *Instituto de Biomedicina de Sevilla IBIS, Hospital Universitario Virgen Macarena/CSIC/Universidad de Sevilla, Seville, Spain*
[3] *Departamento de Microbiología, Universidad de Sevilla, Sevilla, Spain*
[4] *Unidad de Gestión de Farmacia Hospitalaria, Hospital Universitario Virgen Macarena, Seville, Spain*
[5] *Departamento de Farmacología, Universidad de Sevilla, Spain*
[6] *Departamento de Medicina, Universidad de Sevilla, Sevilla, Spain*

A R T I C L E I N F O

Article history:
Received 26 May 2021
Received in revised form
16 September 2021
Accepted 28 September 2021
Available online 8 October 2021

Editor: E. Yusuf

Keywords:
Escherichia coli
Fosfomycin
Fosfomycin activity
Fosfomycin transporters
Inorganic phosphate
Resistance

A B S T R A C T

Objectives: The objective of this study was to evaluate the *in vitro* activity of fosfomycin under different physiological concentrations of inorganic phosphate (P_i).
Methods: The wild-type BW25113 strain, four isogenic mutants (Δ*glpT*, Δ*uhpT*, Δ*glpT-uhpT*, and Δ*phoB*) and six clinical isolates of *Escherichia coli* with different fosfomycin susceptibilities were used. EUCAST breakpoints were used. Susceptibility was evaluated by agar dilution using standard Mueller–Hinton agar (P_i concentration of 1 mM similar to human plasma concentration) and supplemented with P_i (13 and 42 mM, minimum and maximum urinary P_i concentrations) and/or glucose-6-phosphate (25 mg/L). Fosfomycin transporter promoter activity was assayed using P*glpT*::*gfp*mut2 or P*uhpT*::*gfp*mut2 promoter fusions in standard Mueller–Hinton Broth (MHB), supplemented with P_i (13 or 42 mM) ± glucose-6-phosphate. Fosfomycin activity was quantified, estimating fosfomycin EC_{50} under different P_i concentrations (1, 13 and 42 mM + glucose-6-phosphate) and in time–kill assays using fosfomycin concentrations of 307 (maximum plasma concentration (C_{max})), 1053 and 4415 mg/L (urine C_{max} range), using MHB with 28 mM P_i (mean urine P_i concentration) + 25 mg/L glucose-6-phosphate.
Results: All the strains showed decreased susceptibility to fosfomycin linked to increased P_i concentrations: 1–4 $\log_2$ dilution differences from 1 to 13 mM, and 1–8 $\log_2$ dilution differences at 42 mM P_i. Changes in phosphate concentration did not affect the expression of fosfomycin transporters. By increasing P_i concentrations higher fosfomycin EC_{50} bacterial viability was observed, except against Δ*glpT-uhpT*. The increase in P_i reduced the bactericidal effect of fosfomycin.
Discussion: P_i variations in physiological fluids may reduce fosfomycin activity against *E. coli.* Elevated P_i concentrations in urine may explain oral fosfomycin failure in non-wild-type but fosfomycin-susceptible *E. coli* strains. **Miriam Ortiz-Padilla, Clin Microbiol Infect 2022;28:302.e1–302.e4**
© 2021 European Society of Clinical Microbiology and Infectious Diseases. Published by Elsevier Ltd. All rights reserved.

Introduction

Fosfomycin is a bactericidal broad-spectrum antimicrobial, approved for the treatment of uncomplicated urinary tract infections, soft-tissue infections and sepsis caused by Enterobacterales [1]. Fosfomycin uptake is mediated by the uptake systems for *sn*-glycerol-3-phosphate (GlpT) and hexose-6-phosphates (UhpT) via the counterflow of inorganic phosphate

* Corresponding author. Fernando Docobo-Pérez, Departamento de Microbiología, Universidad de Sevilla, Avda. Sánchez Pizjuan s/n., 41009, Sevilla, Spain.
E-mail address: fdocobo1@us.es (F. Docobo-Pérez).
[*] M. Ortiz-Padilla and I. Portillo-Calderón contributed equally as first authors.
[†] F. Docobo-Pérez and A. Pascual contributed equally as senior authors.

https://doi.org/10.1016/j.cmi.2021.09.037
1198-743X/© 2021 European Society of Clinical Microbiology and Infectious Diseases. Published by Elsevier Ltd. All rights reserved.

(P_i) from the cell [2]. This intracellular P_i is maintained within the range 1–10 mM for *Escherichia coli* [3] by P_i importers (PitA, PitB and PstSCAB) and exporters (PitA, PitB, GlpT, UhpT), and controlled by the histidine kinase PhoR and the response regulator PhoB [3]. Human P_i levels vary among the different tissues and fluids, being maintained within a narrow range through a complex interplay between intestinal absorption, exchange with intracellular and bone storage pools, and renal tubular reabsorption [4].

Thus, the aim of the present study was to evaluate the *in vitro* activity of fosfomycin mimicking P_i physiological concentrations against a collection of isogenic *E. coli* mutants and clinical isolates.

Material and methods

Bacterial strains and plasmids

The wild-type *E. coli* BW25113 strain and isogenic mutants (Δ*glpT*, Δ*uhpT* and Δ*phoB*) from the KEIO collection and the double mutant Δ*glpT-uhpT*, generated by phage P1*vir* transduction [5], were used. Six urinary clinical *E. coli* isolates (Ec20190430, Ec20190556, Ec20190736, Ec2019800, Ec20200150 and Ec20200178) with different fosfomycin resistance from the Andalusian reference laboratory for molecular typing of nosocomial pathogens (PIRASOA programme) were included. Bacterial whole genome sequencing was performed as described in the supplementary material. *Escherichia coli* ATCC 25922 was used as control strain for the susceptibility tests. pUA66-P*glpT*::*gfp*mut2 and pUA66-P*uhpT*::*gfp*mut2 promoter fusions were constructed as described by Zaslaver et al. [6].

Bacterial growth medium

Mueller–Hinton Broth (MHB) and Mueller–Hinton Agar (MHA) were used with different concentrations of P_i by adjusting standard Mueller–Hinton medium (P_i = 1 mM, similar to plasma concentration (0.8–1.4 mM)) with Na_2HPO_4 to 13 and 42 mM P_i, corresponding to the minimum and maximum concentrations of P_i in urine, adjusted for a 1-L volume (13–42 mmol/24 hr) or 28 mM (average concentration of P_i in urine) [4].

Susceptibility testing

Fosfomycin MICs were determined by agar dilution [7], in standard MHA (reference) or supplemented with P_i (13 and 42 mM P_i). P_i concentrations in Mueller–Hinton were quantified as described in the supplementary material. EUCAST breakpoints were used for intravenous fosfomycin performed at 1 mM of P_i (i.e. R ≥ 64 mg/L) and for oral fosfomycin at 13 and 42 mM of P_i (i.e. R ≥ 16 mg/L) according to the P_i concentrations in blood and urine, respectively.

Fosfomycin activity

Fosfomycin transporter expressions were assayed by monitoring the fluorescence accumulation in *E. coli* MG1655 carrying pUA66-P*glpT*::*gfp*mut2 or pUA66-P*uhpT*::*gfp*mut2 reporters as described in supplementary data.

Bacterial growth curves were performed in triplicate using 5×10^5 CFU/mL in 96-well plates with 200 μL of volume of standard MHB or supplemented with P_i (13 and 42 mM) with 25 mg/L of glucose-6-phosphate. Fosfomycin concentrations ranged from 1 to 1024 mg/L, and controls without drug were used. Bacterial viability was quantified spectrophotometrically (595 nm) at 24 hr with an Infinite 200 Pro plate reader. Fosfomycin concentration that reduces bacterial viability to 50% (EC_{50}) was estimated by fitting the bacterial viability to the equation: $\text{Viability}(\%) = 100 \div \left(1 + \frac{\text{fosfomycin concentration}}{EC_{50}}\right)$. The mean fosfomycin EC_{50} values were compared using the ANOVA and Tukey and Bonferroni *post hoc* tests.

Time–kill assays were conducted in duplicate, using fosfomycin concentrations of 0 (control), 307 mg/L (plasma C_{max} concentration after 8 g/every 8 hr intravenously) [8], 1053 and 4415 mg/L (lower and upper limit for the mean C_{max} observed in urine after a single dose of 3 g of oral fosfomycin tromethamine) [9], as fully described in the supplementary material.

No ethical considerations were necessary for the present study.

Results

Susceptibility testing

The fosfomycin MICs are shown in Table 1. Increasing MHA P_i concentrations from 1 to 13 mM, 1–4 $\log_2$ dilution differences were observed. Clinical category changes occurred for Δ*glpT* and Ec20190736, Ec20200150 and Ec20200178. The susceptible strains changed to resistant using 42 mM P_i (1–8 $\log_2$ dilution differences).

The Δ*uhpT* and Δ*glpT-uhpT* mutants, Ec20190430 (Δ*glpT-uhpB)* and Ec20190556 were resistant irrespective of the P_i concentration. The isogenic mutant Δ*phoB* and the wild-type strain showed similar MICs at all P_i concentrations tested. Similar results were observed with Δ*glpT* with a 1 $\log_2$ and 2 $\log_2$ increase at P_i 13 and 42 mM, respectively, with respect to the wild-type strain.

Fosfomycin activity

The increased P_i concentration did not cause significant changes in *glpT* or *uhpT* transcription (Fig. S1).

Fosfomycin EC_{50} under increasing P_i concentrations are shown in Table 2 and Fig. S2. Concentration of 13 mM P_i raised the fosfomycin EC_{50} for the wild-type (1.42 vs. 0.34 mg/L) and Δ*glpT* (1.62 vs. 0.32 mg/L) with respect to 1 mM P_i. Concentrations of 42 mM P_i also increased fosfomycin EC_{50} with respect to 1 and 13 mM P_i for the wild-type (8.9 vs. 0.34 and 1.42 mg/L), Δ*glpT* (81.28 vs. 0.32 and 1.62 mg/L) and Δ*uhpT* (65.15 vs. 11.75 and 24.79 mg/L). P_i increased from 1 to 13 mM, showed a 2 $\log_2$ increase in fosfomycin concentration

Table 1
Fosfomycin MICs (mg/L) of the wild-type BW25113, single-gene and double-gene mutants and clinical isolates, tested by agar dilution in MHA with increasing P_i concentrations

Strains	P_i concentration		
	1mM (standard)	13mM	42mM
BW25113 wild type	4 (S)	8 (S)	**64 (R)**
BW25113 Δ*glpT*	4 (S)	**16 (R)**	**256 (R)**
BW25113 Δ*uhpT*	128 (R)	256 (R)	512 (R)
BW25113 Δ*glpT-uhpT*	512 (R)	512 (R)	1024 (R)
BW25113 Δ*phoB*	4 (S)	8 (S)	**128 (R)**
Ec20190430	512 (R)	1024 (R)	1024 (R)
Ec20190556	64 (R)	128 (R)	1024 (R)
Ec20190736	1 (S)	**16 (R)**	**256 (R)**
Ec2019800	2 (S)	8 (S)	**32 (R)**
Ec20200150	8 (S)	**16 (R)**	**64 (R)**
Ec20200178	4 (S)	**16 (R)**	**64 (R)**

Clinical categories for intravenous fosfomycin at 1 mM of P_i (R ≥ 64 mg/L) and oral fosfomycin at P_i 13 and 42 mM (R ≥ 16mg/L) are shown as susceptible (S) or resistant (R). Bold indicates changes in the clinical category.

Table 2
Summary of EC_{50} values calculated from fosfomycin dose–response experiments generated by measuring bacterial viability over 24 hr at different P_i concentrations

P_i concentration	Fosfomycin EC_{50} (95%CI)			
	Wild type	Δ*glpT*	Δ*uhpT*	Δ*glpT-uhpT*
1 mM	0.34 (0.26, 0.42)	0.32 (0.18, 0.48)	11.75 (6.7, 20.74)	260.4 (188.9, 360.5)
13 mM	1.42[a] (1.1, 1.82)	1.62[a] (1.15, 2.25)	24.79 (16.82, 36.41)	334.4 (240.2, 469.3)
42 mM	8.9[a,b](6.24, 12.56)	81.28[a,b](57.63, 114.1)	65.15[a,b](45.51, 92.62)	367.1 (248.4, 549.5)

[a] $p < 0.05$ with respect to 1 mM P_i.
[b] $p < 0.05$ with respect to 13 mM P_i.

able to inhibit bacterial viability, a 4 log_2 dilution increase at 42 mM P_i in the wild-type and Δ*uhpT* strains, and 8 log_2 increase in the Δ*glpT* strain. None of these changes were observed for Δ*glpT-uhpT*. The time–kill assay results (described in the supplementary material) showed that all fosfomycin concentrations exhibited bactericidal activity (>3 log CFU/mL decrease) against the wild-type strain, but fosfomycin activity was more affected in mutants, showing bacteriostatic activity or even bacterial regrowth at higher P_i concentrations.

Discussion

The present study showed a reduction of fosfomycin *in vitro* activity as the P_i concentration increased, focusing on P_i and fosfomycin blood and urine concentrations where should exert its antimicrobial activity. This phenomenon was observed in susceptibility testing, viability and time–kill assays in both the isogenic and clinical isolate collections.

Environmental variables and the presence of different carbon sources have a great impact on fosfomycin activity [10–13], and the importance of phosphate homeostasis in fosfomycin activity was recently highlighted by Turner et al., showing a strong selection of fosfomycin mutants for the phosphonate uptake and catabolism operon and the phosphate transporter [14].

In a previous study aimed at understanding how urinary tract conditions affected fosfomycin activity against *E. coli* strains harbouring chromosomal mutations involved in fosfomycin uptake, urine at pH 7 reduced fosfomycin activity, especially against the null mutants Δ*glpT* and Δ*ptsI* [12]. Among other factors, the different concentrations of P_i could explain, in part, these observations.

Recently, a dynamic bladder infection model simulating oral therapy using MHB, and human and synthetic urine, showed a lack of correlation between fosfomycin MICs determined using agar dilution or broth microdilution with MHB supplemented with glucose-6-phosphate and fosfomycin activity [15], underlining underlines the gap between fosfomycin susceptibility tests and outcome possibly by the relationship between bacterial metabolism and fosfomycin activity.

The main limitation of our *in vitro* study is that Mueller–Hinton composition may have conditioned fosfomycin activity due to the lack of other components present in blood or urine. Also, the limited number of clinical strains with specific genotypes may have biased the conclusions. In conclusion, P_i content modifies fosfomycin activity at concentrations achieved using standard oral or parenteral dosages. Furthermore, Mueller–Hinton media are suitable for fosfomycin susceptibility testing due to their low P_i content, but may overestimate *in vitro* activity with respect to infection sites with higher P_i levels. Both observations should be validated with a larger number of clinical isolates, together with their clinical outcomes especially in cases of P_i homeostasis disorders, to establish the clinical significance.

Transparency declaration

This study has been funded by Instituto de Salud Carlos III through the projects PI16/01824, PI19/01645 and REIPI RD16/0016/0001 (co-funded by European Regional Development Fund "A way to make Europe"). Miriam Ortiz-Padilla is supported by a PFIS grant from the Instituto de Salud Carlos III (FI17/00235). Inés Portillo-Calderón is supported by a Rio Hortega grant from the Instituto de Salud Carlos III (CM20/0092). (Co-funded by European Social Fund "Investing in your future"). The funders had no role in the design, collection of data, analysis and writing of the manuscript or the decision to publish. This study was presented in part at ECCMID 2021 (Abstract number: 00868).

Author contributions

F.D.P., J.M.R.M. and A.P.H. conceived the idea and experimental design. M.O.P., I.P.C. and B.G.I. conducted the phenotypic experiments. M.O.P., I.P.C. N.M.A. and B.G.I. conducted the molecular experiments. V.M.B. quantified P_i concentrations in Mueller–Hinton. F.D.P. and A.P.H. supervised the project. M.O.P., I.P.C., J.M.R.M., J.R.B. and F.D.P. performed the analysis and interpreted the results. F.D.P., A.P.H. and M.O.P. wrote the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2021.09.037.

References

[1] Dijkmans AC, Zacarías NVO, Burggraaf J, Mouton JW, Wilms EB, van Nieuwkoop C, et al. Fosfomycin: pharmacological, clinical and future perspectives. Antibiotics 2017;6:24.
[2] Keseler IM, Mackie A, Santos-Zavaleta A, Billington R, Bonavides-Martínez C, Caspi R, et al. The EcoCyc database: reflecting new knowledge about Escherichia coli K-12. Nucleic Acids Res 2017;45:D543–50.
[3] McCleary WR. Molecular mechanisms of phosphate homeostasis in Escherichia coli. In: Escherichia coli – recent advances on physiology, pathogenesis and biotechnology applications. InTech; 2017. https://doi.org/10.5772/67283.
[4] Goldman L, Schafer A. Goldman–Cecil Medicine. 26th ed. New York: Elsevier; 2019.
[5] Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, et al. Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol 2006;2. 2006.0008.
[6] Zaslaver A, Bren A, Ronen M, Itzkovitz S, Kikoin I, Shavit S, et al. A comprehensive library of fluorescent transcriptional reporters for Escherichia coli. Nat Methods 2006;3:623–8.

[7] The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 11.0. 2021. http://www.eucast.org.
[8] Pfausler B. Concentrations of fosfomycin in the cerebrospinal fluid of neuro-intensive care patients with ventriculostomy-associated ventriculitis. J Antimicrob Chemother 2004;53:848–52.
[9] Patel SS, Balfour JA, Bryson HM. Fosfomycin tromethamine. A review of its antibacterial activity, pharmacokinetic properties and therapeutic efficacy as a single-dose oral treatment for acute uncomplicated lower urinary tract infections. Drugs 1997;53:637–56.
[10] Kahan FM, Kahan JS, Cassidy PJ, Kropp H. The mechanism of action of fosfomycin (phosphonomycin). Ann N Y Acad Sci 1974;235:364–86.
[11] Kurabayashi K, Tanimoto K, Fueki S, Tomita H, Hirakawa H. Elevated expression of GlpT and UhpT via FNR activation contributes to increased fosfomycin susceptibility in Escherichia coli under anaerobic conditions. Antimicrob Agents Chemother 2015;59:6352–60.
[12] Martin-Gutiérrez G, Docobo-Pérez F, Rodriguez-Beltŕn J, Rodriguez-Martinez JM, Aznar J, Pascual A, et al. Urinary tract conditions affect fosfomycin activity against Escherichia coli strains harboring chromosomal mutations involved in fosfomycin uptake. Antimicrob Agents Chemother 2017;62. e01899-17.
[13] Xavier KB, Kossmann M, Santos H, Boos W. Kinetic analysis by in vivo 31P nuclear magnetic resonance of internal P_i during the uptake of sn-glycerol-3-phosphate by the pho regulon-dependent Ugp system and the glp regulon-dependent GlpT system. J Bacteriol 1995;177:699–704.
[14] Turner AK, Yasir M, Bastkowski S, Telatin A, Page AJ, Charles IG, et al. A genome-wide analysis of Escherichia coli responses to fosfomycin using TraDIS-Xpress reveals novel roles for phosphonate degradation and phosphate transport systems. J Antimicrob Chemother 2020;75:3144–51.
[15] Abbott IJ, van Gorp E, Wijma RA, Meletiadis J, Mouton JW, Peleg AY. Evaluation of pooled human urine and synthetic alternatives in a dynamic bladder infection in vitro model simulating oral fosfomycin therapy. J Microbiol Methods 2020;171:105861.

Supplementary Material of Role of inorganic phosphate concentrations in in vitro activity of fosfomycin.

MATERIAL AND METHODS

Bacterial growth medium

Ion chromatography, using Metrohm 883 basic IC plus equipment with a Metrosep A supp 5 column and precolumn (Metrohm Hispania, Madrid, Spain), was carried out to determine Pi concentrations in standard Mueller Hinton media. Pi was identified by comparing the retention time established in the conditions of analysis with a certified standard, and quantified using a 7-point calibration line with R_2=0.999 and average relative standard deviation of 1.943%, ≤ 5% in any case**.**

Mueller-Hinton Broth (MHB) and Mueller-Hinton Agar (MHA) were used with different concentrations of Pi by supplementing standard Mueller-Hinton medium (Pi=1mM) with Na_2HPO_4 until the desired concentrations of 13 and 42mM of Pi were reached, corresponding to the minimum and maximum concentrations of Pi in urine, adjusted for a 1L volume (13-42mmol/24h) (1), and 28mM, corresponding to the average concentration of Pi in urine.

Fosfomycin-transporters induction

Fosfomycin transporter expressions were assayed by monitoring the fluorescence accumulation in *E. coli* MG1655 carrying pUA66-P*glpT*::*gfp*mut2 or pUA66-P*uhpT*::*gfp*mut2 reporters. Bacterial growth curves were performed using $5x10_5$CFU/mL as starting inoculum in 96-well plates with 200µL of volume per well. MHB 1mM Pi (standard), MHB 13Mm or 42Mm Pi were used. Glucose-6-phosphate (25mg/L) was added as control for *uhpT* gene expression. Green fluorescence (excited at 485nm and measured at 540nm) and bacterial growth (measured at 595nm) were monitored at 0 and 24 hours with an Infinite200 Pro plate reader (Tecan Group AG, Männedorf, Switzerland). Assays were performed in quintuplicate. Fluorescence was normalized to OD and the background was subtracted. The OD:fluorescence ratio of the promoterless construction (pUA66-Ø::*gfp*mut2) was used as background for all experiments under the different growth conditions. The results were compared with ANOVA and Tukey and Bonferroni post-hoc tests.

Time-kill assays.

Time-kill assays were conducted in duplicate, using fosfomycin concentrations of 0 (control), 307mg/L (Cmax concentration in plasma after an intravenous dose of 8g/Q8h) (2), 1053 and 4415mg/L (lower and upper limit for the mean Cmax observed in urine after a single dose of 3g of oral fosfomycin tromethamine) (3). The assay was done in standard MHB or supplemented with Pi (28mM, mean Pi urine concentration), with 25mg/L glucose-6-phosphate. Bacterial inoculum was

$5x10^5$CFU/mL in 20mL. The number of viable CFUs, after incubation with shaking at 37ºC, was determined at 0, 2, 4, 8, and 24h by serial dilution followed by plating on MHA plates.

RESULTS

Whole-genome sequencing

Analyses of translated nucleotide sequences of genes associated with fosfomycin resistance or regulation (Table S1) showed no mutations in Crp and UhpA proteins in our collection of clinical isolates. No deletions or insertions were detected in the rest of the amino acid sequences associated with fosfomycin resistance, although many different polymorphisms were found, some of them conserved in most clinical isolates, except for the Ec20190430 isolate (fosfomycin MIC of 512 mg/L), which showed deletions in GlpT and UhpB, and Ec20190178 (fosfomycin MIC of 4 mg/L), which presented a deletion causing a frameshift mutation and a premature stop codon. With respect to proteins involved in the bacterial phosphate regulatory system (Table S2), only PitA and PhoB showed WT sequences. The rest of the amino acid sequences likewise showed many polymorphisms, some of them conserved in the clinical isolates. Nevertheless, the biological significance of many of the observed polymorphisms is unclear.

Fosfomycin transport induction

Peak fosfomycin transporter promoter activities (at 4h), evaluated at Pi concentrations of 1, 13 and 42mM ±25mg/L of glucose-6-phosphate, are shown in figure S1. The increased Pi concentration did not cause significant changes in *glpT* or *uhpT* transcription. Only the addition of glucose-6-phosphate significantly increased *uhpT* transcription.

Time-kill assay results

All fosfomycin concentrations showed bactericidal activity (>3log CFU/mL decrease) against the wild-type strain. It should be noted that 307mg/L of fosfomycin was unable to clear the bacterial culture at 28mM Pi. Similar results were observed in the Δ*glpT* strain at the low Pi concentration. At 28mM Pi, however, bacterial regrowth occurred at fosfomycin concentrations of 307 and 1053mg/L.

With respect to the Δ*uhpT* strain, the bacterial counts dropped in the first 4h at all fosfomycin concentrations, although regrowth was observed at 307mg/L of fosfomycin after 8h with both Pi concentrations, and at 1053mg/L of fosfomycin with 28mM Pi. Finally, against the double mutant (Δ*glpT-uhpT*), not even 4015mg/L of fosfomycin was able to sterilise the bacterial culture with 28mM Pi after 24h. However, early fosfomycin activity was observed at 1053 and 4015mg/L with

both Pi concentrations, although bacterial regrowth was observed with the lowest fosfomycin concentration and a static effect with the highest.

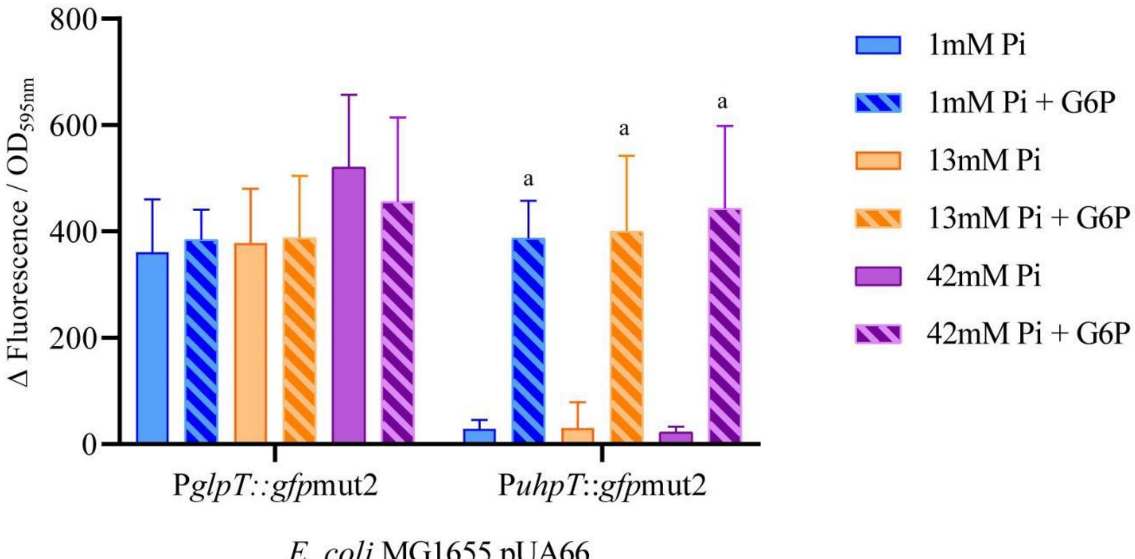
Table S1. Amino acid modifications in fosfomycin-related resistance proteins relative to reference strain BW25113. *del* = deletion.

Strains	GlpT	GlpR	UhpT	UhpB	UhpC	GlpK	CyaA	PtsI	MurA
BW25113									
BW Δ*glpT*	*del*								
BW Δ*uhpT*			*del*						
BWΔ*glpT*-*uhpT*	*del*		*del*						
BW ΔphoB									
Ec 20190430	p.210_246*del*			p.307_379*del*	Y18H			R367K	
Ec 20190556		P233Q		Q377K	Y18H		N142S	L6V / S51L / R367K / T437I	
Ec 20190736			E35Q	P84S / Q441H / G459D / Q463H / H482T	Y18H / A177S / S417A	L121M / D123E / S127N	N142S / A349E / S356K / G359E / EA362-363DS / D837E / T840A	R367K	
Ec 20190800									P99S
Ec 20200150							G683D		
Ec 20200178	E448K		E350Q		Y18H / A177S / S417A		N141S / HNK712-714QVN / p715-848STOP*del*	R367K	

Table S2. Amino acid modifications in genes related to phosphate uptake relative to reference strain BW25113. del = deletion.

Strains	UgpB	UgpA	UgpE	UgpC	UgpQ	PitB	PhoB	PhoR	PstS
BW25113									
BW Δ*glpT*									
BW Δ*uhpT*									
BWΔ*glpT*-*uhpT*									
BW ΔphoB							*del*		
Ec 20190430			V252A		S26A / M88A / E135Q /Q158L / N196D / A198V	A41T / S333T			G147D / S243L / I285V / T296V
Ec 20190556			V252A	A341P	S26A / M88A / E135Q / A160V / N196D /A198V	A41T / F63L			
Ec 20190736	H5R / G19A / T371I	S71A	A40E / Q140K / V252A	E58T / NDQ64-66DRK / D152E / T244A / E261T / M297L / A341P	S26A / M88A / M128T / A138T / N196D	A41T / Y180F / T299A		N230D	
Ec 2019800			V252A		S26A				
Ec 20200150			V252A		S26A	A41T / Y180F / T299A			
Ec 20200178			Q140K / V252A	M297L	S26A / M88A / E135Q / Q158L / N196D / A198V	A41T / S333T		L19F / N230D / H272Q / Q344L / P363L	

Figure S1. Promoter activity of fosfomycin transporters *glpT* and *uhpT* at different concentrations of Pi. Glucose-6-phosphate (G6P, 25mg/L) was used as positive control for *uhpT* expression. Bars and whiskers indicate mean and standard deviation.



a P<0.05 with respect to the absence of G6P.

Figure S2. Viability after 24h of wild-type *E. coli* BW25113 and isogenic mutant strains (Δ*glpT* , Δ*uhpT* and Δ*glpT-uhpT*) against fosfomycin concentrations ranging from 0-1024 mg/L in standard MHB (Pi=1 mM) and supplemented with Pi (final concentration of 13 and 42mM). Lines and symbols indicate mean values (±SD) of viability measurements.

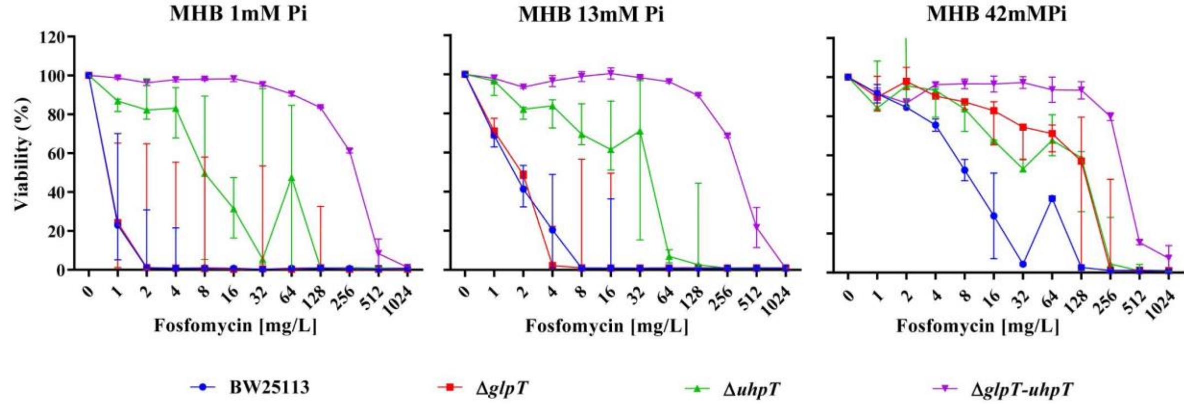
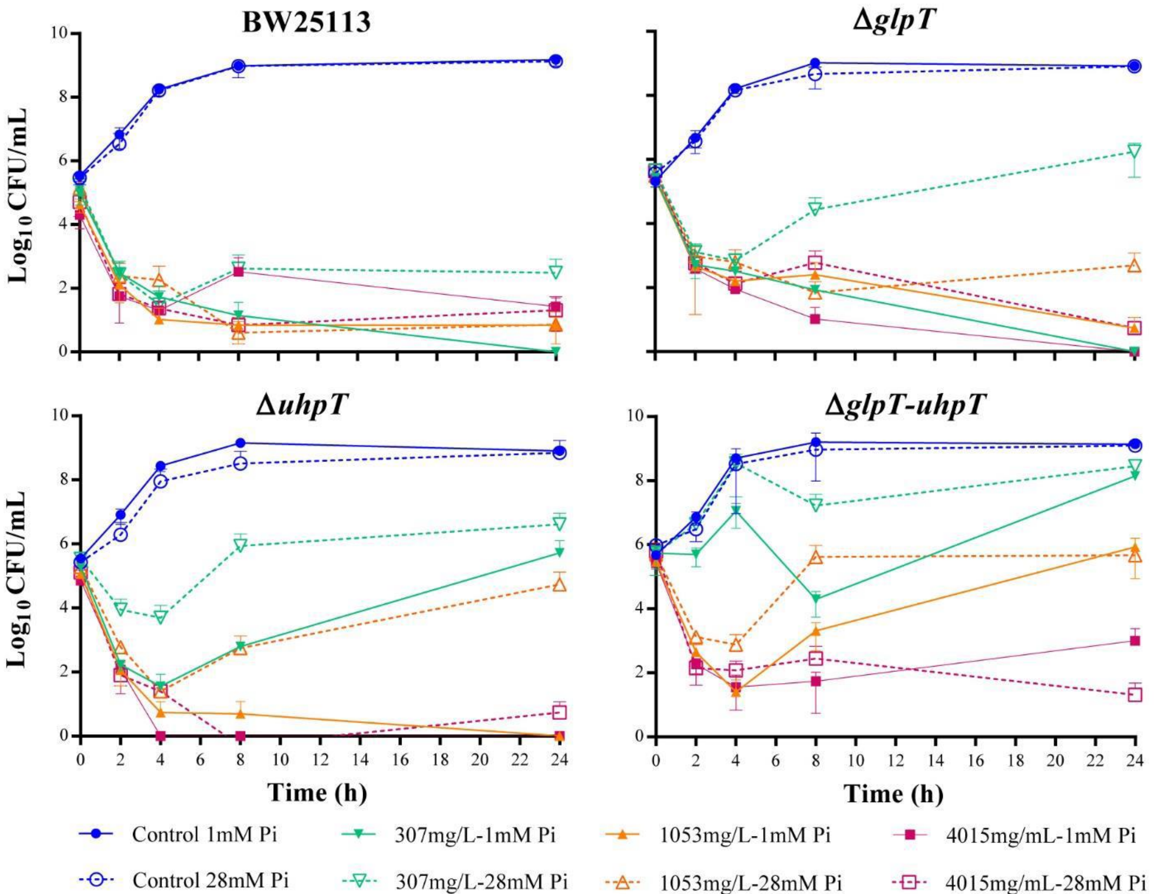


Figure S3. 24-hour time-kill results for wild-type *E. coli* BW25113 and isogenic mutant strains (Δ*glpT* , Δ*uhpT* and Δ*glpT-uhpT*) using 307, 1053 and 4015 mg/L fosfomycin in standard MHB (Pi=1 mM, continuous lines) and supplemented with Pi (final Pi concentration of 28mM, dashed lines) .



REFERENCES

1. Goldman L (Columbia U, Schafer A (Weill CMC. Goldman-Cecil Medicine 26th Edition. Elsevier. New York; 2019. 2944 p.

2. Pfausler B. Concentrations of fosfomycin in the cerebrospinal fluid of neurointensive care patients with ventriculostomy-associated ventriculitis. J Antimicrob Chemother [Internet]. 2004 Mar 24;53(5):848–52. Available from: http://www.jac.oupjournals.org/cgi/doi/10.1093/jac/dkh158

3. Patel SS, Balfour JA, Bryson HM. Fosfomycin tromethamine. A review of its antibacterial activity, pharmacokinetic properties and therapeutic efficacy as a single-dose oral treatment for acute uncomplicated lower urinary tract infections. Drugs. 1997 Apr;53(4):637–56.

VI. DISCUSSION

The progressive increase in antimicrobial resistance is a worldwide public health problem that science is trying to find a solution under different ways[18]. The current global interest in the recovery of old antimicrobials and the optimization of their use for the treatment of infections caused by multidrug-resistant microorganisms is the result of the scarcity of therapeutic alternatives. This strategy has been proposed by international organizations such as the WHO or the ECDC, focusing in certain antimicrobials [21,80,225].

These old antimicrobials have remained active and in some cases with low resistance profiles over the years due to their low use, motivated by the emergence of more promising drugs[80].

In this sense, fosfomycin is an antimicrobial discovered more than 50 years ago and is still active against bacteria with multiple resistance mechanisms[194]. Thus, the systemic use of this antibiotic belongs to the options in the therapeutic arsenal used against infections caused by multidrug resistant bacteria according to the EMA[83].

Despite the availability of this drug for several decades, its use as intravenous formulation has been limited until the last decade. One of the restricting factors in the use of this drug has been the peculiarities and controversies in the development and interpretation of the susceptibility studies, a key factor for the prediction of the therapeutic success or failure of an antimicrobial treatment[226,227]. In this sense, the CLSI and EUCAST committees establish that the reference method for determining- the susceptibility or resistance to fosfomycin is the dilution in agar supplemented with G6P (25 mg/L)[201,228]. However, this procedure is unfeasible for its development in the daily routine of the clinical microbiology laboratory, for this reason the use of better adapted, easier, and faster alternative methodologies (disc-diffusion, broth microdilution, or gradient strips) have been adopted by the laboratories. These adaptations from the reference method differ and originates discrepant susceptibility data depending on the methodology used[227,229]. This produces uncertainty in the extrapolation of the results into the clinic. This may have contributed to some extent to the generation of some mistrust in the use of this drug as a first-line systemic treatment.

Considering the potential relevance of this antimicrobial as a potential alternative use for systemic infections caused by multidrug resistant bacteria, it is essential to understand the factors that can alter its mechanism of action, like how fosfomycin is transported into the bacteria, how they are regulated (a main step obtain its full activity), or whether there are molecules that can optimize and improve fosfomycin activity. All this, aimed to be able to predict the efficacy of the drug in a more adequate and individualized way and to increase the chance of therapeutic success.

In the first presented work, we evaluated the impact of specific determinants in the resistance to fosfomycin, present in *K. pneumoniae;* including transporter defects, as well as the contribution of chromosomal metalloenzymes such as *fosA*. The impact of fosfomycin-inactivating enzymes on fosfomycin resistance among *Enterobacterales* has been widely studied from different perspectives[230,231]. In our study, we constructed a collection of null mutants focused on the elimination fosfomycin inactivating-enzyme (*fosA*) gen, and the fosfomycin transporters *glpT* and *uhpT,* to evaluated whether the use of a compound able to reduce or inactivate its activity against fosfomycin could be of clinical interest against fosfomycin susceptible and resistant strains.

Therefore, this work studied the impact of fosfomycin resistance determinants in *K. pneumoniae* clinical isolates during *in vitro* exposure to fosfomycin in combination with sodium phosphonoformate (PPF), a FosA inhibitor. In our mutant isogenic collection, the fosfomycin susceptibility of the wild-type *K. pneumoniae* isolates was 32mg/L. Theses results agree with the most frequent MIC observed in EUCAST epidemiological MIC distribution[209]. The strains with the *glpT* null mutant did not cause an increase in fosfomycin resistance, compared to wild-type strains. This phenotype is consistent with that previously observed in *E. coli* susceptibility assays [165,232]. These results are explained by the overexpression of *uhpT* transporter produced by the addition of G6P to the susceptibility, assay according to the EUCAST and CLSI guidelines, that masks the presence of mutations no related with the Uhp transporter system[165]. On the other hand, *uhpT* inactivation significantly increased the MIC of fosfomycin (≥1,024 mg/L). This observation can be explained by the limited uptake of fosfomycin via UhpT together with inactivation of fosfomycin mediated by chromosomally mediated FosA. These results differ from that previously observed in *E. coli* but can be easily explained by the absence of the inactivating enzyme in this bacterial specie. In the case of inactivation of the *fosA* gene, a reduction in resistance was produced of approximately 32-fold compared to that of the wild-type strains (1 mg/L). These results match with the most frequent MIC observed in EUCAST epidemiological MIC distribution in *E. coli* indicating the similarities between *K. pneumoniae* and *E. coli* when the chromosomal *fosA* is inactive[172,209]. These results open door to explore a pharmacological approach aimed to inactivate FosA.

With respect to the assays with the addition of PPF, the experiments showed a variable effect on the susceptibility tests. The addition of PPF increased fosfomycin susceptibility against in the wild-type and *glpT* null mutants. These results agree with those previously observed by *Ito et al.,* where incorporation of PPF reduced fosfomycin MIC (≥4-fold) for 52% of *E. coli*, *K. pneumoniae*, *Enterobacter cloacae* and *P. aeruginosa* clinical isolates [176]. However, in all cases the addition of PPF showed less decrease of fosfomycin resistance compared to that observed with the deletion of the *fosA* gene. It is important to note that no effect was found in the null mutant for *uhpT* gene. An interesting finding was

observed with the addition of PPF to the *fosA* null mutant, where the fosfomycin MIC increased with the combination of the inhibitor. Because PPF inhibits viral RNA polymerases, reverse transcriptase, and DNA polymerases through non-competitive inhibition with dNTPs[177], a mutagenic role of this compound had to be discarded. However, no variations in the mutant frequency assays were observed when PPF was added to the detection of rifampicin-resistant mutants. Thus, this discrepancy cannot be explained by an increment in the mutant frequency caused by PPF. Another possible explanation, not tested in this work, could be that PPF would be transported by UhpT, competing with fosfomycin for being intracellularly transported. In the subsequent studies regarding the pharmacological approach to inhibit FosA activity, a concentration range study combining fosfomycin and PPF was carried out to assess the interaction between fosfomycin and PPF in terms of synergy or antagonism. The maximum synergistic area was observed within the fosfomycin susceptibility range (from 2 to 8 mg/L for *K. pneumoniae* ATCC 700721 and from 8 to 32 mg/L fosfomycin for Kp12 and Kp142). This synergistic activity was also found to be favourable with PPF in a range of 0.3 to 2.5 mM, corresponding to human blood concentrations after dosing 90 mg/kg body weight every 12 h (q12h) (mean ± standard deviation [SD] for the maximum steady-state concentration of the drug in serum [Cmax], 0.623±0.132 mM)[233]. In this sense, *Ito et al.* reported similar results using agar dilution assays, in which they obtained an apparent low activity of PPF at concentrations up to 0.667 mM against some isolates with high MIC values of fosfomycin[176].

In line with the previous results, the growth monitoring assays demonstrated higher fosfomycin activity against the *fosA* null mutant, relative to the wild-type strain. Despite this enhanced activity with the addition PPF, again these results did not provide an improvement over those results obtained with the Δ*fosA* strains. These discrepancies might be explained by lack of effective PPF activity blocking FosA or by a degradation or inactivation of PPF during the assay.

With respect to the strains with deleted *glpT*, fosfomycin activity resulted lower than that of wild-type strains, although they had similar MICs. These results agree with a higher proportion of fosfomycin mutants observed with the Δ*glpT* strains with respect to the wild-type strain. These observations were consistent with those reported previously by our group in *E. coli*[165], showing that highly resistant mutants emerged from Δ*glpT* strains after selection for additional mutations at loci associated with fosfomycin resistance. No higher activity was observed against *uhpT* null mutants when the assays were supplemented with PPF. Similar results were observed with the remaining clinical isolates. In strains with low MICs and in the absence of fosfomycin resistance mutations, an effect of the addition of PPF could be observed, whereas in strains with high MIC or with mutations, the addition of PPF did not have an effect.

Finally, the time-kill assays revealed results similar to those found in the growth assays. The inactivation of *fosA* considerably improved the efficacy of fosfomycin, with reductions in bacterial the burden of 1-5$\log_{10}$ CFU/mL. The addition of PPF to the assays mildly improved fosfomycin activity but only within the first hours except for the *uhpT* mutant. However, at the end of the assay highly resistant mutants emerged in all the conditions. Our study differs slightly from the results obtained by Ito *et al.*[176]. In that study, using a single strain of *K. pneumoniae* with a fosfomycin MIC of 256 mg/L, the authors found a bactericidal effect at 8 and 24 h using concentrations of 256 and 512 mg/L, but a bacterial regrowth at 24 h with 128 mg/L. The addition of PPF to this assay showed a bactericidal effect at all concentrations of fosfomycin tested, including 128 mg/L.

The present work concludes that strategies aimed at inactivating *fosA* activity by gene editing or using pharmacological approaches are potentially promising for increasing the activity of fosfomycin against strains of *K. pneumoniae* [234]. In this regard, the pharmacological approach is the most feasible, but more PK/PD models should be conducted to better evaluate the activity of FosA-inactivating compounds.

In the line of optimizing the use of fosfomycin through the use in combination with molecules that can increase it activity, the second manuscript of the present Thesis evaluates the role of glycerol as an enhancer of fosfomycin activity. This improvement would occur increasing fosfomycin uptake through the activation of GlpT transporter.

The work shows that, under therapeutically relevant concentrations of glycerol, the *glpT* promoter can be activated, increasing its expression[137,235]. This result agrees with previous work showing the activation of genes (*glpACB*, *glpD*, *glpFKX*, *glpTQ*, etc.) that are implicated in glycerol catabolism and regulated by the *glpR* repressor in *E. coli*, *P. aeruginosa* or *P. putida*[138–140]. However, this induction of *glpT* by glycerol exhibits a delayed onset, that contrast to the faster activation of the hexose-6-phosphate transporter (*uhpT*) after the addition of G6P. These differences in the rate of transporter induction have been reported using GFP promoter fusions in real-time fluorescence monitoring and flow cytometry assays for the *glpT* and *uhpT* genes.

Nikel et al. proposed an explanation for this behaviour, where a prolonged lag phase in *P. putida* KT2440 was observed in cultures grown in glycerol as sole carbon source [139]. The *glp* gene regulatory network requires G3P, a product of glycerol phosphorylation, to derepress gene expression, which is otherwise inhibited by GlpR. However, the genes that encode the glycerol transporter and the kinase that produces G3P are also repressed by the GlpR protein. Thus, to initiate the transcription process, a stochastic repression lifting is required, which has low probability and is activator independent. This stochastic derepression process produces different levels of metabolic activity (representing, in this

context, the ability of cells to catabolize glycerol). This *glp* regulation system is highly conserved in different species like *E. coli, K. pneumoniae, P. aeruginosa*, *P. putida, etc.*, although not all have the same genes (*P. putida* does not have the *glpT* gene)[138,164,165].

Regarding the activity of fosfomycin in combination with glycerol, this study showed heterogeneous results depending on the characteristics of the assay. In the disc diffusion assays, it was observed that the addition of glycerol increased susceptibility for in wild-type phenotype strains and all mutants except Δ*glpT*-*uhpT*, Δ*glpR*, and Δ*glpK*. However, secondary and reduced zones of inhibition were also detected, indicating the presence of subpopulations with different susceptibility to fosfomycin, even with the use of glycerol and G6P in combination. The maximum fosfomycin activity was observed against the *glpR* repressor mutant and the addition of glycerol did not increase its activity. This is explained, because in under the absence of repressor the expression of the *glpT* transporter is fully derepressed. Furthermore, the addition of glycerol did not show any effect on the *glpK* null mutant indicating the role of kinase in the generation of intracellular glycerol-3-phosphate[235]. However, fosfomycin activity was similar to that observed against the wild-type *E. coli* MG1655 strain in the absence of glycerol, and this could be explained in part by the intracellular production of sn-glycerol-3-phosphate via UgpQ, GpsA, Gpr, etc., as a precursor of phospholipid synthesis[236]. The totally absence of activity was observed in the *ΔglpT-uhpT* double gene mutant strain, pointing out the activation of fosfomycin transporters as the main factor controlling fosfomycin activity, as previously observed by *Ballesteros et al*[165]. in *E. coli.* It is also important to note that the reduced inhibition zones remained unchanged regardless of the combination with glycerol or G6P, suggesting that this phenotype was not affect by these molecules, enhancing the complex regulatory networks of the fosfomycin uptake and activity [107,109].

In this study, discrepancies between susceptibility testing by disc diffusion and agar dilution were observed. These results agree with our previous studies, in which fosfomycin susceptibility showed inconsistent results between broth and agar dilutions and agar diffusion techniques in collections of clinical isolates, fosfomycin heteroresistant strains or fosfomycin resistance-related isogenic mutants[165,227,232]. Although the addition of glycerol did not modify fosfomycin MIC, performed with the agar dilution method, it should be noted that lower bacterial densities were observed, indicating a mild synergistic effect that could not be measurable with this kind of assay. In the fosfomycin-glycerol interaction assays, synergistic activity was observed only for the wild-type and Δ*uhpT* mutant strains of *E. coli* MG1655. However, the time-kill assays did not reflect this sensitization with the combination of fosfomycin and glycerol. It is important to note that in time-kill assays, divergent results between replicates were found for wild-type and *E. coli* MG1655 Δ*uhpT* strains. In the case of these strains, one replicate showed total eradication of the bacterial burden and the other

bacterial regrew after 24 hours. This partially matched with the results seen in the interaction assay. This observed divergence between the replicates could be explained in view of the rapid bactericidal activity of fosfomycin combined with the delayed activation of the *glpT* transporter. This challenge increases the probability of the emergence of subpopulations that may have variable resistance to fosfomycin, as observed in previous studies[165,237].

In conclusion, glycerol showed an increased expression of the fosfomycin transporter *glpT* and a synergistic effect with fosfomycin in interaction and disc diffusion assays. However, this molecule does not appear to be a good candidate as an adjuvant to be added to a fosfomycin treatment, as the delay in induction of the *glpT* transporter allows the selection of resistant subpopulations. Therefore, further *in vitro* and *in vivo* studies would be necessary to overcome these problems.

Following the line of study of the importance of fosfomycin transporters, the third publication analysed a physiological factor that may alter the activity of the transporters and thus the activity of the antimicrobial. The following work evaluated the role of inorganic phosphate (Pi) concentrations on fosfomycin activity. The inorganic phosphate is the molecule expelled by GlpT and UhpT proteins when they enter their natural substrates or fosfomycin they its present. Environmental variables like oxygen, Pi concentration, pH, and the presence of different carbon sources such as G6P, G3P and glycerol, among others, have a great impact on fosfomycin activity [123,126,238–240]. In this, context, different studies have shown inhibition of G3P or G6P intake by different metabolites, including elevated Pi concentrations[126,240]. The importance of phosphate homeostasis in fosfomycin activity was also recently highlighted in a genome-wide analysis of *E. coli* in response to fosfomycin, using transposon-directed insertion site sequencing that combines an inducible promoter into the transposon cassette (TraDIS-Xpress) [241]. Turner *et al.* showed strong selection of fosfomycin mutants for the phosphonate uptake and catabolism operon (*phnCDEFGHILJLMNO*) and the phosphate transporter, PstBCAS.

For the development of our study a collection of isogenic *E. coli* mutants for *glpT*, *uhpT* and *phoB* from BW25113 and six clinical isolates was used, to evaluate the influence of Pi concentration on fosfomycin activity, focusing on blood (1mM) and urine concentrations (13-42mM) where fosfomycin should exert its antimicrobial activity. Fosfomycin intake in *Enterobacterales* occurs mainly through GlpT and UhpT transporters [107]. Like other proteins, the two transporters act as Pi exporters not directly regulated by the main Pi homeostasis controller in *E. coli,* the Pho regulon, in agreement with our studies regarding the activity of the fosfomycin transport induction results, in which the increase in phosphate concentration did not cause significant changes in transcription of these transporters [242]In a previous study aimed at understanding how urinary tract conditions affected fosfomycin activity against *E. coli* strains harbouring chromosomal mutations involved in fosfomycin uptake, urine at pH=7 reduced fosfomycin activity, especially against the null mutants Δ*glpT* and Δ*ptsI* [239]. Among other

factors, the different concentrations of Pi could explain, in part, these observations, in line with our work, where the main result was the reduction of antimicrobial activity as the Pi concentration increased. This phenomenon was observed in susceptibility testing, fosfomycin activity assays and time-kill assays in both the isogenic and clinical isolate collections.

Almost all the time-kill results showed that there is a possibility of regrowth at physiological concentrations of fosfomycin and Pi (urine conditions), in strains with mutations for fosfomycin transports, or if the concentration of fosfomycin remains low. Once again, this underline the gap between fosfomycin susceptibility tests and outcome due to the close relationship between bacterial metabolism and fosfomycin activity, in line with other studies as recent dynamic bladder infection model simulating oral therapy using MHB supplemented with G6P, and human and synthetic urine, there was a lack of correlation between fosfomycin MICs determined using agar dilution or broth microdilution with MHB supplemented with G6P and fosfomycin activity[243].

The main limitation of our study is that the *in vitro* assays were carried out in MHB and the lack of other nutrients or factors that may be present in blood or urine may have conditioned fosfomycin activity. However, the low Pi content of MHB offers a well-known background medium for the study of a single variable such as Pi concentration, rather than using minimal media where bacteria often show poor growth and are not recommended for susceptibility testing. In addition, the limited number of clinical strains based on the inclusion of specific genotypes of fosfomycin resistance may have biased the conclusions.

In conclusion, Pi content modifies fosfomycin activity at concentrations achieved using standard oral or parenteral dosages. Furthermore, Mueller-Hinton media are suitable for fosfomycin susceptibility testing due to their low Pi content but may overestimate *in vitro* activity with respect to infection sites with higher inorganic phosphate levels. Both observations should be validated with a larger number of clinical isolates, together with their clinical outcomes, to establish the clinical significance.

VII. CONCLUSIONS

1. The inactivation of the *fosA* gene show decreased fosfomycin resistance in *K. pneumoniae* clinical isolates, leading to a susceptibility level similar to that found in *E. coli*.
2. The pharmacological approach with sodium phosphonoformate did not achieve enough activity and the effect decreased with the presence of fosfomycin resistance mutations.
3. Glycerol induces transcription of the GlpT transporter, has a synergistic effect in combination with fosfomycin and increases fosfomycin susceptibility in wild-type and mutant strains such as in the UhpT transporter gene at therapeutically relevant concentrations.
4. Glycerol does not appear to be a suitable molecule for use as an adjuvant together with fosfomycin, as the induction of the *glpT* transporter is slow, resulting in emergence of resistant subpopulations.
5. Extracellular high inorganic phosphate concentration increases fosfomycin resistance.
6. This increase is not mediated by direct regulation of GlpT and UhpT transporters and occurs at physiological inorganic phosphate concentrations.

VIII. BIBLIOGRAPHY

1. Aminov, R. I. A brief history of the antibiotic era: lessons learned and challenges for the future. *Front. Microbiol.* **1**, (2010).
2. *Uso y abuso de los antibióticos. ¿Dónde estamos y adonde queremos llegar?* (2006).
3. CDC. 2019 Antibiotic Resistance Threats Report | CDC. https://www.cdc.gov/drugresistance/biggest-threats.html (2019).
4. Das, B., Chaudhuri, S., Srivastava, R., Nair, G. B. & Ramamurthy, T. Fostering research into antimicrobial resistance in India. *BMJ* **358**, j3535 (2017).
5. Clemente, J. C. *et al.* The microbiome of uncontacted Amerindians. *Sci. Adv.* **1**, (2015).
6. Bhullar, K. *et al.* Antibiotic resistance is prevalent in an isolated cave microbiome. *PLoS One* **7**, (2012).
7. Davies, J., Spiegelman, G. B. & Yim, G. The world of subinhibitory antibiotic concentrations. *Curr. Opin. Microbiol.* **9**, 445–453 (2006).
8. Fajardo, A. & Martínez, J. L. Antibiotics as signals that trigger specific bacterial responses. *Curr. Opin. Microbiol.* **11**, 161–167 (2008).
9. Aminov, R. I. The role of antibiotics and antibiotic resistance in nature. *Environ. Microbiol.* **11**, 2970–2988 (2009).
10. Perry, J. A., Westman, E. L. & Wright, G. D. The antibiotic resistome: what's new? *Curr. Opin. Microbiol.* **21**, 45–50 (2014).
11. Kim, D. W. & Cha, C. J. Antibiotic resistome from the One-Health perspective: understanding and controlling antimicrobial resistance transmission. **53**, 301–309 (2021).
12. Partridge, S. R., Kwong, S. M., Firth, N. & Jensen, S. O. Mobile Genetic Elements Associated with Antimicrobial Resistance. *Clin. Microbiol. Rev.* **31**, (2018).
13. Cox, G. & Wright, G. D. Intrinsic antibiotic resistance: mechanisms, origins, challenges and solutions. *Int. J. Med. Microbiol.* **303**, 287–292 (2013).
14. Frère, J.-M., Sauvage, E. & Kerff, F. From 'An Enzyme Able to Destroy Penicillin' to Carbapenemases: 70 Years of Beta-lactamase Misbehaviour. *Curr. Drug Targets* **17**, 974–982 (2016).
15. J, D. & D, D. Origins and evolution of antibiotic resistance. *Microbiol. Mol. Biol. Rev.* **74**, 9–16 (2010).
16. Hiltunen, T., Virta, M. & Anna-Liisa, L. Antibiotic resistance in the wild: an eco-evolutionary perspective. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **372**, (2017).
17. Baquero, F., Tedim, A. P. & Coque, T. M. Antibiotic resistance shaping multi-level population biology of bacteria. *Front. Microbiol.* **4**, (2013).
18. O'Neil, J. Tackling drug-resistant infection globally: final report and recommendations the review on antimicrobial resistant chaired by Jim O'Neil. (2016).
19. Cassini, A. *et al.* Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. **19**, 56–66 (2019).

20. Falagas, M. E., Tansarli, G. S., Karageorgopoulos, D. E. & Vardakas, K. Z. Deaths attributable to carbapenem-resistant Enterobacteriaceae infections. *Emerg. Infect. Dis.* **20**, 1170–1175 (2014).

21. WHO. WHO Library Cataloguing-in-Publication Data Global Action Plan on Antimicrobial Resistance. *Microbe Mag.* **10**, 354–355 (2015).

22. Mathers, A. J., Peirano, G. & Pitout, J. D. D. The role of epidemic resistance plasmids and international high-risk clones in the spread of multidrug-resistant Enterobacteriaceae. *Clin. Microbiol. Rev.* **28**, 565–591 (2015).

23. Carattoli, A. Plasmids and the spread of resistance. *Int. J. Med. Microbiol.* **303**, 298–304 (2013).

24. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *Lancet (London, England)* **399**, 629–655 (2022).

25. *European Centre for Disease Prevention and Control. Antimicrobial resistance in the EU/EEA (EARS-Net) - Annual Epidemiological Report 2021. Stockholm: ECDC; 2022.*

26. Daneman, N. *et al.* Variability in Antibiotic Use Across Nursing Homes and the Risk of Antibiotic-Related Adverse Outcomes for Individual Residents. *JAMA Intern. Med.* **175**, 1331–1339 (2015).

27. Klein, E. Y. *et al.* Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. *Proc. Natl. Acad. Sci. U. S. A.* **115**, E3463–E3470 (2018).

28. WHO. Monitoring and evaluation of the global action plan on antimicrobial resistance: framework and recommended indicators. https://apps.who.int/iris/handle/10665/325006 (2021).

29. ISCIII (Instituto de Salud Carlos III). *Memoria 2020 de la Red de vigilancia EARS-Net*. (2020).

30. Robinson, T. P. *et al.* Antibiotic resistance is the quintessential One Health issue. *Trans. R. Soc. Trop. Med. Hyg.* **110**, 377–380 (2016).

31. WHO. The evolving threat of antimicrobial resistance: options for action. https://apps.who.int/iris/handle/10665/44812 (2014).

32. ECDC, E. C. for D. P. and C. Antimicrobial resistance in the EU/EEA (EARS-Net) - Annual Epidemiological Report for 2020. https://www.ecdc.europa.eu/en/publications-data/antimicrobial-resistance-eueea-ears-net-annual-epidemiological-report-2020 (2020).

33. AEMPS. Plan estratégico y de acción para reducir el riesgo de selección y diseminación de la resistencia a los antibioticos. (2014).

34. Storr, J. *et al.* Core components for effective infection prevention and control programmes: new WHO evidence-based recommendations. *Antimicrob. Resist. Infect. Control* **6**, (2017).

35. Tacconelli, E. *et al.* ESCMID guidelines for the management of the infection control measures to reduce transmission of multidrug-resistant Gram-negative bacteria in hospitalized patients. *Clin. Microbiol. Infect.* **20 Suppl 1**, 1–55 (2014).

36. Aiello, A. E., Coulborn, R. M., Perez, V. & Larson, E. L. Effect of hand hygiene on infectious disease risk in the community setting: a meta-analysis. *Am. J. Public Health* **98**, 1372–1381 (2008).

37. Vikesland, P. *et al.* Differential Drivers of Antimicrobial Resistance across the World. *Acc. Chem. Res.* **52**, 916–924 (2019).

38. Bürgmann, H. *et al.* Water and sanitation: an essential battlefront in the war on antimicrobial resistance. *FEMS Microbiol. Ecol.* **94**, (2018).

39. Dellit, T. H. *et al.* Infectious Diseases Society of America and the Society for Healthcare Epidemiology of America guidelines for developing an institutional program to enhance antimicrobial stewardship. *Clin. Infect. Dis.* **44**, 159–177 (2007).

40. Rodríguez-Baño, J. *et al.* Programas de optimización de uso de antimicrobianos (PROA) en hospitales españoles: documento de consenso GEIH-SEIMC, SEFH y SEMPSPH. *Enferm. Infecc. Microbiol. Clin.* **30**, 22.e1-22.e23 (2012).

41. Barlam, T. F. The state of antibiotic stewardship programs in 2021: The perspective of an experienced steward. *Antimicrob. Steward. Healthc. Epidemiol. ASHE* **1**, (2021).

42. Schuts, E. C. *et al.* Current evidence on hospital antimicrobial stewardship objectives: a systematic review and meta-analysis. *Lancet. Infect. Dis.* **16**, 847–856 (2016).

43. OECD. *Stemming the Superbug Tide*. https://www.oecd-ilibrary.org/social-issues-migration-health/stemming-the-superbug-tide_9789264307599-en (2018) doi:10.1787/9789264307599-EN.

44. Servicio Andaluz de Salud. *Programa integral de prevención, control de las infecciones relacionadas con la asistencia sanitaria, y uso apropiado de los antimicrobianos.* https://www.juntadeandalucia.es/servicioandaluzdesalud/ (2014).

45. Cantas, L. *et al.* A brief multi-disciplinary review on antimicrobial resistance in medicine and its linkage to the global environmental microbiota. *Front. Microbiol.* **4**, (2013).

46. MOORE, P. R. & EVENSON, A. Use of sulfasuxidine, streptothricin, and streptomycin in nutritional studies with the chick. *J. Biol. Chem.* **165**, 437–441 (1946).

47. Anderson, A. D., Nelson, J. M., Rossiter, S. & Angulo, F. J. Public health consequences of use of antimicrobial agents in food animals in the United States. *Microb. Drug Resist.* **9**, 373–379 (2003).

48. Góchez, D. *et al.* OIE Annual Report on Antimicrobial Agents Intended for Use in Animals: Methods Used. *Front. Vet. Sci.* **6**, (2019).

49. Muloi, D. *et al.* Are Food Animals Responsible for Transfer of Antimicrobial-Resistant Escherichia coli or Their Resistance Determinants to Human Populations? A Systematic Review. *Foodborne Pathog. Dis.* **15**, 467–474 (2018).

50. (EMA), E. medicines A. Sales of veterinary antimicrobial agents in 31 European countries in 2019 and 2020. (2021) doi:10.2809/167341.

51. Walsh, T. R. & Wu, Y. China bans colistin as a feed additive for animals. *Lancet. Infect. Dis.* **16**, 1102–1103 (2016).

52. Liu, Y. Y. *et al.* Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet. Infect. Dis.* **16**, 161–168 (2016).

53. Van Boeckel, T. P. *et al.* Reducing antimicrobial use in food animals. *Science* **357**, 1350–1352 (2017).

54. World Health Organisation (WHO). Critically Important Antimicrobials for Human Medicine 6th Re.vision 2018. Ranking of medically important antimicrobials for risk management of antimicrobial resistance due to non-human use. *Https://Apps.Who.Int/Iris/Bitstream/Handle/10665/312266/9789241515528-Eng.Pdf?Ua=1* ISBN 978-92-4-151552-8 (2018).

55. (FAO), F. and agriculture organization. The FAO Action Plan on Antimicrobial Resistance 2016-2020. https://www.fao.org/fsnforum/resources/reports-and-briefs/fao-action-plan-antimicrobial-resistance-2016-2020 (2021).

56. Lewis, K. Platforms for antibiotic discovery. *Nat. Rev. Drug Discov. 2013 125* **12**, 371–387 (2013).

57. Singer, A. C., Kirchhelle, C. & Roberts, A. P. (Inter)nationalising the antibiotic research and development pipeline. *Lancet. Infect. Dis.* **20**, e54–e62 (2020).

58. Tacconelli, E. *et al.* Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *Lancet. Infect. Dis.* **18**, 318–327 (2018).

59. WHO. 2019 antibacterial agents in clinical development: an analysis of the antibacterial clinical development pipeline. https://www.who.int/publications/i/item/9789240000193 (2019).

60. The Pew Charitable Trust. Tracking the Global Pipeline of Antibiotics in Development, March 2021 | The Pew Charitable Trusts. https://www.pewtrusts.org/en/research-and-analysis/issue-briefs/2021/03/tracking-the-global-pipeline-of-antibiotics-in-development (2021).

61. Niu, G. & Li, W. Next-Generation Drug Discovery to Combat Antimicrobial Resistance. *Trends Biochem. Sci.* **44**, 961–972 (2019).

62. Czaplewski, L. *et al.* Alternatives to antibiotics-a pipeline portfolio review. *Lancet. Infect. Dis.* **16**, 239–251 (2016).

63. Yang, J. H., Bening, S. C. & Collins, J. J. Antibiotic efficacy-context matters. *Curr. Opin. Microbiol.* **39**, 73–80 (2017).

64. Bush, K. & Bradford, P. A. β-Lactams and β-Lactamase Inhibitors: An Overview. *Cold Spring Harb. Perspect. Med.* **6**, (2016).

65. DIckey, S. W., Cheung, G. Y. C. & Otto, M. Different drugs for bad bugs: antivirulence strategies in the age of antibiotic resistance. *Nat. Rev. Drug Discov.* **16**, 457–471 (2017).

66. Kaufmann, S. H. E., Dorhoi, A., Hotchkiss, R. S. & Bartenschlager, R. Host-directed therapies for bacterial and viral infections. *Nat. Rev. Drug Discov.* **17**, 35–56 (2018).

67. Lee, H. H. & Collins, J. J. Microbial environments confound antibiotic efficacy. *Nat. Chem. Biol.* **8**, 6–9 (2011).

68. Farha, M. A. & Brown, E. D. Drug repurposing for antimicrobial discovery. *Nat. Microbiol.* **4**, 565–577 (2019).

69. Santacroce, L., Charitos, I. A. & Bottalico, L. A successful history: probiotics and their potential as antimicrobials. *Expert Rev. Anti. Infect. Ther.* **17**, 635–645 (2019).

70. Golkar, Z., Bagasra, O. & Gene Pace, D. Bacteriophage therapy: a potential solution for the antibiotic resistance crisis. *J. Infect. Dev. Ctries.* **8**, 129–136 (2014).

71. Vázquez-Ucha, J. C., Arca-Suárez, J., Bou, G. & Beceiro, A. New Carbapenemase Inhibitors: Clearing the Way for the β-Lactams. *Int. J. Mol. Sci.* **21**, 1–32 (2020).

72. Tomich, A. D. *et al.* Small-Molecule Inhibitor of FosA Expands Fosfomycin Activity to Multidrug-Resistant Gram-Negative Pathogens. *Antimicrob. Agents Chemother.* **63**, (2019).

73. Savage, P. B. Multidrug-resistant bacteria: overcoming antibiotic permeability barriers of gram-negative bacteria. *Ann. Med.* **33**, 167–171 (2001).

74. Blanco, P., Sanz-García, F., Hernando-Amado, S., Martínez, J. L. & Alcalde-Rico, M. The

development of efflux pump inhibitors to treat Gram-negative infections. *Expert Opin. Drug Discov.* **13**, 919–931 (2018).

75. Antunes, L. C. M., Ferreira, R. B. R., Buckner, M. M. C. & Finlay, B. B. Quorum sensing in bacterial virulence. *Microbiology* **156**, 2271–2282 (2010).

76. Stokes, J. M., Lopatkin, A. J., Lobritz, M. A. & Collins, J. J. Bacterial Metabolism and Antibiotic Efficacy. *Cell Metab.* **30**, 251–259 (2019).

77. Liu, Y., Li, R., Xiao, X. & Wang, Z. Bacterial metabolism-inspired molecules to modulate antibiotic efficacy. *J. Antimicrob. Chemother.* **74**, 3409–3417 (2019).

78. de la Fuente-Núñez, C., Reffuveille, F., Haney, E. F., Straus, S. K. & Hancock, R. E. W. Broad-spectrum anti-biofilm peptide that targets a cellular stress response. *PLoS Pathog.* **10**, (2014).

79. Kottur, J. & Nair, D. T. Reactive Oxygen Species Play an Important Role in the Bactericidal Activity of Quinolone Antibiotics. *Angew. Chem. Int. Ed. Engl.* **55**, 2397–2400 (2016).

80. Zayyad, H., Eliakim-Raz, N., Leibovici, L. & Paul, M. Revival of old antibiotics: needs, the state of evidence and expectations. *Int. J. Antimicrob. Agents* **49**, 536–541 (2017).

81. Theuretzbacher, U. *et al.* Reviving old antibiotics. *J. Antimicrob. Chemother.* **70**, 2177–2181 (2015).

82. Mouton, J. W. *et al.* Conserving antibiotics for the future: new ways to use old and new drugs from a pharmacokinetic and pharmacodynamic perspective. *Drug Resist. Updat.* **14**, 107–117 (2011).

83. Karaiskos, I., Lagou, S., Pontikis, K., Rapti, V. & Poulakou, G. The 'Old' and the 'New' Antibiotics for MDR Gram-Negative Pathogens: For Whom, When, and How. *Front. public Heal.* **7**, (2019).

84. Sheu, C. C., Chang, Y. T., Lin, S. Y., Chen, Y. H. & Hsueh, P. R. Infections Caused by Carbapenem-Resistant Enterobacteriaceae: An Update on Therapeutic Options. *Front. Microbiol.* **10**, (2019).

85. López-Montesinos, I. & Horcajada, J. P. Oral and intravenous fosfomycin in complicated urinary tract infections. *Rev. Esp. Quimioter.* **32 Suppl 1**, 37–44 (2019).

86. Muller, A. E., Verhaegh, E. M., Harbarth, S., Mouton, J. W. & Huttner, A. Nitrofurantoin's efficacy and safety as prophylaxis for urinary tract infections: a systematic review of the literature and meta-analysis of controlled trials. *Clin. Microbiol. Infect.* **23**, 355–362 (2017).

87. Hendlin, D. *et al.* Phosphonomycin, a new antibiotic produced by strains of streptomyces. *Science* **166**, 122–123 (1969).

88. Shoji, J. *et al.* Production of fosfomycin (phosphonomycin) by Pseudomonas syringae. *J. Antibiot. (Tokyo).* **39**, 1011–1012 (1986).

89. Cameron, S., McLuskey, K., Chamberlayne, R., Hallyburton, I. & Hunter, W. N. Initiating a crystallographic analysis of recombinant (S)-2-hydroxypropylphosphonic acid epoxidase from Streptomyces wedmorensis. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* **61**, 534 (2005).

90. Kim, S. Y. *et al.* Different Biosynthetic Pathways to Fosfomycin in Pseudomonas syringae and Streptomyces Species. *Antimicrob. Agents Chemother.* **56**, 4175 (2012).

91. Cao, Y., Peng, Q., Li, S., Deng, Z. & Gao, J. The intriguing biology and chemistry of fosfomycin: the only marketed phosphonate antibiotic. *RSC Adv.* **9**, 42204 (2019).

92. Kahan, F. M., Kahan, J. S., Cassidy, P. J. & Kropp, H. The mechanism of action of fosfomycin (phosphonomycin). *Ann. N. Y. Acad. Sci.* **235**, 364–386 (1974).

93. Andrews, J. M. *et al.* International collaborative study on standardization of bacterial sensitivity to fosfomycin. *J. Antimicrob. Chemother.* **12**, 357–361 (1983).

94. Grimm, H. In vitro investigations with fosfomycin on Mueller-Hinton agar with and without glucose-6-phosphate. *Infection* **7**, 256–259 (1979).

95. Bergan, T. Degree of absorption, pharmacokinetics of fosfomycin trometamol and duration of urinary antibacterial activity. *Infection* **18 Suppl 2**, (1990).

96. Gupta, K. *et al.* International clinical practice guidelines for the treatment of acute uncomplicated cystitis and pyelonephritis in women: A 2010 update by the Infectious Diseases Society of America and the European Society for Microbiology and Infectious Diseases. *Clin. Infect. Dis.* **52**, (2011).

97. EMA. *Recommendations to restrict use of fosfomycin antibiotics | European Medicines Agency*. https://www.ema.europa.eu/en/news/recommendations-restrict-use-fosfomycin-antibiotics (2020).

98. Nabriva Therapeutics Resubmits New Drug Application for Intravenous CONTEPO^{TM} (fosfomycin) for Injection | Nabriva Therapeutics. https://investors.nabriva.com/news-releases/news-release-details/nabriva-therapeutics-resubmits-new-drug-application-intravenous (2019).

99. Soledad Pérez, D., Pérez, D., Tapia, M. & Soraci, A. Fosfomycin: Uses and potentialities in veterinary medicine. *Open Vet. J.* **4**, 26 (2014).

100. Gobernado, M. A.-Sociedad Española de Quimioterapia Revisión Fosfomicina. *Marzo* **16**, 15–40 (2003).

101. Cao, Y., Peng, Q., Li, S., Deng, Z. & Gao, J. The intriguing biology and chemistry of fosfomycin: the only marketed phosphonate antibiotic. *RSC Adv.* **9**, 42204–42218 (2019).

102. Kahan, F. M., Kahan, J. S., Cassidy, P. J. & Kropp, H. The mechanism of action of fosfomycin (phosphonomycin). *Ann. N. Y. Acad. Sci.* **235**, 364–386 (1974).

103. Falagas, M. E., Vouloumanou, E. K., Samonis, G. & Vardakas, K. Z. Fosfomycin. *Clin. Microbiol. Rev.* **29**, 321 LP – 347 (2016).

104. Brown, E. D., Vivas, E. I., Walsh, C. T. & Kolter, R. MurA (MurZ), the enzyme that catalyzes the first committed step in peptidoglycan biosynthesis, is essential in Escherichia coli. *J. Bacteriol.* **177**, 4194–4197 (1995).

105. Skarzynski, T. *et al.* Structure of UDP-N-acetylglucosamine enolpyruvyl transferase, an enzyme essential for the synthesis of bacterial peptidoglycan, complexed with substrate UDP-N-acetylglucosamine and the drug fosfomycin. *Structure* **4**, 1465–1474 (1996).

106. Eschenburg, S., Priestman, M. & Schönbrunn, E. Evidence that the fosfomycin target Cys115 in UDP-N-acetylglucosamine enolpyruvyl transferase (MurA) is essential for product release. *J. Biol. Chem.* **280**, 3757–3763 (2005).

107. Castañeda-García, A., Blázquez, J. & Rodríguez-Rojas, A. Molecular mechanisms and clinical impact of acquired and intrinsic fosfomycin resistance. *Antibiotics* **2**, 217–236 (2013).

108. Vardakas, K. Z., Legakis, N. J., Triarides, N. & Falagas, M. E. Susceptibility of contemporary isolates to fosfomycin: a systematic review of the literature. *Int. J. Antimicrob. Agents* **47**, 269–285 (2016).

109. Marger, M. D. & Saier, M. H. A major superfamily of transmembrane facilitators that catalyse uniport, symport and antiport. *Trends Biochem. Sci.* **18**, 13–20 (1993).

110. Ambudkar, S. V., Larson, T. J. & Maloney, P. C. Reconstitution of sugar phosphate transport systems of Escherichia coli. *J. Biol. Chem.* **261**, 9083–9086 (1986).

111. Grainger, D. C., Hurd, D., Harrison, M., Holdstock, J. & Busby, S. J. W. Studies of the distribution of Escherichia coli cAMP-receptor protein and RNA polymerase along the E. coli chromosome. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 17693 (2005).

112. Zubay, G., Schwartz, D. & Beckwith, J. Mechanism of activation of catabolite-sensitive genes: a positive control system. *Proc. Natl. Acad. Sci. U. S. A.* **66**, 104–110 (1970).

113. Franchini, A. G., Ihssen, J. & Egli, T. Effect of Global Regulators RpoS and Cyclic-AMP/CRP on the Catabolome and Transcriptome of Escherichia coli K12 during Carbon- and Energy-Limited Growth. *PLoS One* **10**, (2015).

114. Balsalobre, C., Johansson, J. & Uhlin, B. E. Cyclic AMP-dependent osmoregulation of crp gene expression in Escherichia coli. *J. Bacteriol.* **188**, 5935–5944 (2006).

115. Sakamoto, Y., Furukawa, S., Ogihara, H. & Yamasaki, M. Fosmidomycin resistance in adenylate cyclase deficient (cya) mutants of Escherichia coli. *Biosci. Biotechnol. Biochem.* **67**, 2030–2033 (2003).

116. Görke, B. & Stülke, J. Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. *Nat. Rev. Microbiol.* **6**, 613–624 (2008).

117. Deutscher, J. The mechanisms of carbon catabolite repression in bacteria. *Curr. Opin. Microbiol.* **11**, 87–93 (2008).

118. Fann, M. C. *et al.* Identification of two essential arginine residues in UhpT, the sugar phosphate antiporter of Escherichia coli. *J. Membr. Biol.* **164**, 187–195 (1998).

119. Lloyd, A. D. & Kadner, R. J. Topology of the Escherichia coli uhpT sugar-phosphate transporter analyzed by using TnphoA fusions. *J. Bacteriol.* **172**, 1688–1693 (1990).

120. Kadner, R. J. & Shattuck Eidens, D. M. Genetic control of the hexose phosphate transport system of Escherichia coli: mapping of deletion and insertion mutations in the uhp region. *J. Bacteriol.* **155**, 1052–1061 (1983).

121. Island, M. D. & Kadner, R. J. Interplay between the membrane-associated UhpB and UhpC regulatory proteins. *J. Bacteriol.* **175**, 5028–5034 (1993).

122. Chen, Q. & Kadner, R. J. Effect of altered spacing between uhpT promoter elements on transcription activation. *J. Bacteriol.* **182**, 4430–4436 (2000).

123. Kurabayashi, K., Tanimoto, K., Fueki, S., Tomita, H. & Hirakawa, H. Elevated expression of GlpT and UhpT via FNR activation contributes to increased fosfomycin susceptibility in Escherichia coli under anaerobic conditions. *Antimicrob. Agents Chemother.* **59**, 6352–6360 (2015).

124. Kurabayashi, K., Hirakawa, Y., Tanimoto, K., Tomita, H. & Hirakawa, H. Role of the CpxAR two-component signal transduction system in control of fosfomycin resistance and carbon substrate uptake. *J. Bacteriol.* **196**, 248–256 (2014).

125. Verhamme, D. T., Postma, P. W., Crielaard, W. & Hellingwerf, K. J. Cooperativity in signal transfer through the Uhp system of Escherichia coli. *J. Bacteriol.* **184**, 4205–4210 (2002).

126. Xavier, K. B., Kossmann, M., Santos, H. & Boos, W. Kinetic analysis by in vivo 31P nuclear magnetic resonance of internal Pi during the uptake of sn-glycerol-3-phosphate by the pho regulon-dependent Ugp system and the glp regulon-dependent GlpT system. *J. Bacteriol.* **177**, 699–704 (1995).

127. Ortiz-Padilla, M. *et al.* Role of inorganic phosphate concentrations in in vitro activity of fosfomycin. *Clin. Microbiol. Infect.* **28**, 302.e1-302.e4 (2022).

128. Fann, M. C. & Maloney, P. C. Functional symmetry of UhpT, the sugar phosphate transporter of Escherichia coli. *J. Biol. Chem.* **273**, 33735–33740 (1998).

129. Grimm, H. In vitro investigations with fosfomycin on Mueller-Hinton agar with and without glucose-6-phosphate. *Infection* **7**, 256–259 (1979).

130. Huang, Y., Lemieux, M. J., Song, J., Auer, M. & Wang, D. N. Structure and mechanism of the glycerol-3-phosphate transporter from Escherichia coli. *Science* **301**, 616–620 (2003).

131. Lemieux, M. J., Huang, Y. & Wang, D. N. Crystal structure and mechanism of GlpT, the glycerol-3-phosphate transporter from E. coli. *J. Electron Microsc. (Tokyo).* **54**, i43–i46 (2005).

132. Cole, S. T. *et al.* Nucleotide sequence and gene-polypeptide relationships of the glpABC operon encoding the anaerobic sn-glycerol-3-phosphate dehydrogenase of Escherichia coli K-12. *J. Bacteriol.* **170**, 2448–2456 (1988).

133. Yang, B., Gerhardt, S. G. & Larson, T. J. Action at a distance for glp repressor control of glpTQ transcription in Escherichia coli K-12. *Mol. Microbiol.* **24**, 511–521 (1997).

134. Yang, B. & Larson, T. J. Multiple promoters are responsible for transcription of the glpEGR operon of Escherichia coli K-12. *Biochim. Biophys. Acta* **1396**, 114–126 (1998).

135. Iuchi, S., Cole, S. T. & Lin, E. C. C. Multiple regulatory elements for the glpA operon encoding anaerobic glycerol-3-phosphate dehydrogenase and the glpD operon encoding aerobic glycerol-3-phosphate dehydrogenase in Escherichia coli: further characterization of respiratory control. *J. Bacteriol.* **172**, 179–184 (1990).

136. Truniger, V., Boos, W. & Sweet, G. Molecular analysis of the glpFKX regions of Escherichia coli and Shigella flexneri. *J. Bacteriol.* **174**, 6981–6991 (1992).

137. Lin, E. C. Glycerol dissimilation and its regulation in bacteria. *Annu. Rev. Microbiol.* **30**, 535–78 (1976).

138. Koch, J. P., Hayashi, S. & Lin, E. C. C. The Control of Dissimilation of Glycerol and l-α-Glycerophosphate in Escherichia coli. *J. Biol. Chem.* **239**, 3106–3108 (1964).

139. Nikel, P. I., Romero-Campero, F. J., Zeidman, J. A., Goñi-Moreno, Á. & de Lorenzo, V. The glycerol-dependent metabolic persistence of Pseudomonas putida KT2440 reflects the regulatory logic of the GlpR repressor. *MBio* **6**, (2015).

140. Nikel, P. I., Kim, J. & de Lorenzo, V. Metabolic and regulatory rearrangements underlying glycerol metabolism in Pseudomonas putidaKT2440. *Environ. Microbiol.* **16**, 239–254 (2014).

141. Freedberg, W. B. & Lin, E. C. C. Three kinds of controls affecting the expression of the glp regulon in Escherichia coli. *J. Bacteriol.* **115**, 816–823 (1973).

142. Takahata, S. *et al.* Molecular mechanisms of fosfomycin resistance in clinical isolates of Escherichia coli. *Int. J. Antimicrob. Agents* **35**, 333–337 (2010).

143. Santoro, A. *et al.* Interaction of fosfomycin with the Glycerol 3-phosphate Transporter of Escherichia coli. *Biochim. Biophys. Acta - Gen. Subj.* **1810**, 1323–1329 (2011).

144. McCleary, W. R. Molecular Mechanisms of Phosphate Homeostasis in Escherichia coli. in *Escherichia coli - Recent Advances on Physiology, Pathogenesis and Biotechnological Applications* (InTech, 2017). doi:10.5772/67283.

145. Hsieh, Y.-J. & Wanner, B. L. Global regulation by the seven-component Pi signaling system. *Curr. Opin. Microbiol.* **13**, 198–203 (2010).

146. Gardner, S. G. & McCleary, W. R. Control of the phoBR Regulon in Escherichia coli. *EcoSal Plus* **8**, (2019).

147. Harris, R. M., Webb, D. C., Howitt, S. M. & Cox, G. B. Characterization of PitA and PitB fromEscherichia coli *J. Bacteriol.* **183**, 5008 LP – 5014 (2001).

148. Elvin, C. M., Hardy, C. M. & Rosenberg, H. P(i) exchange mediated by the GlpT-dependent sn-glycerol-3-phosphate transport system in Escherichia coli. *J. Bacteriol.* **161**, 1054–1058 (1985).

149. Ambudkar, S. V., Anantharam, V. & Maloney, P. C. UhpT, the sugar phosphate antiporter of Escherichia coli, functions as a monomer. *J. Biol. Chem.* **265**, 12287–12292 (1990).

150. Motomura, K. *et al.* Overproduction of YjbB reduces the level of polyphosphate in Escherichia coli: A hypothetical role of YjbB in phosphate export and polyphosphate accumulation. *FEMS Microbiol. Lett.* **320**, 25–32 (2011).

151. Kumar, S., Parvathi, A., Hernandez, R. L., Cadle, K. M. & Varela, M. F. Identification of a novel UDP-N-acetylglucosamine enolpyruvyl transferase (MurA) from Vibrio fischeri that confers high fosfomycin resistance in Escherichia coli. *Arch. Microbiol.* **191**, 425–429 (2009).

152. De Smet, K. A. L., Kempsell, K. E., Gallagher, A., Duncan, K. & Young, D. B. Alteration of a single amino acid residue reverses fosfomycin resistance of recombinant MurA from Mycobacterium tuberculosis. *Microbiology* **145 (Pt 11)**, 3177–3184 (1999).

153. McCoy, A. J., Sandlin, R. C. & Maurelli, A. T. In vitro and in vivo functional activity of Chlamydia MurA, a UDP-N-acetylglucosamine enolpyruvyl transferase involved in peptidoglycan synthesis and fosfomycin resistance. *J. Bacteriol.* **185**, 1218–1228 (2003).

154. Gil-Marqués, M. L. *et al.* Peptidoglycan recycling contributes to intrinsic resistance to fosfomycin in Acinetobacter baumannii. *J. Antimicrob. Chemother.* **73**, 2960–2968 (2018).

155. Borisova, M., Gisin, J. & Mayer, C. Blocking peptidoglycan recycling in Pseudomonas aeruginosa attenuates intrinsic resistance to fosfomycin. *Microb. Drug Resist.* **20**, 231–237 (2014).

156. Gisin, J., Schneider, A., Nägele, B., Borisova, M. & Mayer, C. A cell wall recycling shortcut that bypasses peptidoglycan de novo biosynthesis. *Nat. Chem. Biol.* **9**, 491–493 (2013).

157. Scortti, M. *et al.* Coexpression of virulence and fosfomycin susceptibility in Listeria: molecular basis of an antimicrobial in vitro-in vivo paradox. *Nat. Med.* **12**, 515–517 (2006).

158. Li, X. *et al.* Abrp, a new gene, confers reduced susceptibility to tetracycline, glycylcine, chloramphenicol and fosfomycin classes in Acinetobacter baumannii. *Eur. J. Clin. Microbiol. Infect. Dis.* **35**, 1371–1375 (2016).

159. Venkateswaran, P. S. & Wu, H. C. Isolation and characterization of a phosphonomycin-resistant mutant of Escherichia coli K-12. *J. Bacteriol.* **110**, 935–944 (1972).

160. Ohkoshi, Y. *et al.* Mechanism of reduced susceptibility to fosfomycin in Escherichia coli clinical isolates. *Biomed Res. Int.* **2017**, (2017).

161. Couce, A. *et al.* Genomewide overexpression screen for fosfomycin resistance in Escherichia coli: MurA confers clinical resistance at low fitness cost. *Antimicrob. Agents Chemother.* **56**, 2767–2769 (2012).

162. Lee, Y. C., Chen, P. Y., Wang, J. T. & Chang, S. C. Prevalence of fosfomycin resistance and gene

mutations in clinical isolates of methicillin-resistant Staphylococcus aureus. *Antimicrob. Resist. Infect. Control* **9**, (2020).

163. Kurabayashi, K., Tanimoto, K., Tomita, H. & Hirakawa, H. Cooperative Actions of CRP-cAMP and FNR Increase the Fosfomycin Susceptibility of Enterohaemorrhagic Escherichia coli (EHEC) by Elevating the Expression of glpT and uhpT under Anaerobic Conditions. *Front. Microbiol.* **8**, 426 (2017).

164. Castañeda-García, A., Rodríguez-Rojas, A., Guelfo, J. R. & Blázquez, J. The Glycerol-3-Phosphate Permease GlpT Is the Only Fosfomycin Transporter in Pseudomonas aeruginosa. *J. Bacteriol.* **191**, 6968–6974 (2009).

165. Ballestero-Téllez, M. *et al.* Molecular insights into fosfomycin resistance in Escherichia coli. *J. Antimicrob. Chemother.* **72**, 1303–1309 (2017).

166. Nilsson, A. I., Berg, O. G., Aspevall, O., Kahlmeter, G. & Andersson, D. I. Biological costs and mechanisms of fosfomycin resistance in Escherichia coli. *Antimicrob. Agents Chemother.* **47**, 2850–2858 (2003).

167. Li, Y. *et al.* Antimicrobial Susceptibility and Molecular Mechanisms of Fosfomycin Resistance in Clinical Escherichia coli Isolates in Mainland China. *PLoS One* **10**, e0135269 (2015).

168. Kobayashi, S., Kuzuyama, T. & Seto, H. Characterization of the fomA and fomB gene products from Streptomyces wedmorensis, which confer fosfomycin resistance on Escherichia coli. *Antimicrob. Agents Chemother.* **44**, 647–650 (2000).

169. Walkty, A. *et al.* Fosfomycin resistance mediated by fos genes remains rare among extended-spectrum beta-lactamase-producing Escherichia coli clinical isolates recovered from the urine of patients evaluated at Canadian hospitals (CANWARD, 2007-2017). *Diagn. Microbiol. Infect. Dis.* **96**, (2020).

170. Cao, M., Bernat, B. A., Wang, Z., Armstrong, R. N. & Helmann, J. D. FosB, a cysteine-dependent fosfomycin resistance protein under the control of sigma(W), an extracytoplasmic-function sigma factor in Bacillus subtilis. *J. Bacteriol.* **183**, 2380–2383 (2001).

171. Fillgrove, K. L., Pakhomova, S., Schaab, M. R., Newcomer, M. E. & Armstrong, R. N. Structure and mechanism of the genomically encoded fosfomycin resistance protein, FosX, from Listeria monocytogenes. *Biochemistry* **46**, 8110–8120 (2007).

172. Ito, R. *et al.* Widespread Fosfomycin Resistance in Gram-Negative Bacteria Attributable to the Chromosomal fosA Gene. *MBio* **8**, e00749-17 (2017).

173. Zurfluh, K., Treier, A., Schmitt, K. & Stephan, R. Mobile fosfomycin resistance genes in Enterobacteriaceae-An increasing threat. *Microbiologyopen* **9**, (2020).

174. Biggel, M., Zurfluh, K., Treier, A., Nü Esch-Inderbinen, M. & Stephan, R. Characteristics of fosA-carrying plasmids in E. coli and Klebsiella spp. isolates originating from food and environmental samples. doi:10.1093/jac/dkab119.

175. Yang, T. Y., Lu, P. L. & Tseng, S. P. Update on fosfomycin-modified genes in Enterobacteriaceae. *J. Microbiol. Immunol. Infect.* **52**, 9–21 (2019).

176. Ito, R. *et al.* Inhibition of fosfomycin resistance protein FosA by phosphonoformate (foscarnet) in multidrug-resistant gram-negative pathogens. *Antimicrob. Agents Chemother.* **61**, (2017).

177. Rigsby, R. E., Rife, C. L., Fillgrove, K. L., Newcomer, M. E. & Armstrong, R. N. Phosphonoformate: A minimal transition state analogue inhibitor of the fosfomycin resistance protein, FosA. *Biochemistry* **43**, 13666–13673 (2004).

178. Nakamura, G. *et al.* Practical Agar-Based Disk Potentiation Test for Detection of Fosfomycin-Nonsusceptible Escherichia Coli Clinical Isolates Producing Glutathione S-transferases. *J. Clin. Microbiol.* **52**, 3175 LP – 3179 (2014).

179. Falagas, M. E. *et al.* Antimicrobial susceptibility of Gram-positive non-urinary isolates to fosfomycin. *Int. J. Antimicrob. Agents* **35**, 497–499 (2010).

180. EUCAST. EUCAST intrinsic resistance and unusual phenotypes. (2020).

181. Khan, I. U. *et al.* In vitro activity of fosfomycin tromethamine against extended spectrum beta-lactamase producing urinary tract bacteria. *J. Coll. Physicians Surg. Pak.* **24**, 914–917 (2014).

182. Maraki, S. *et al.* In vitro susceptibility and resistance phenotypes in contemporary Citrobacter isolates in a University Hospital in Crete, Greece. *Infect. Dis. (London, England)* **49**, 532–539 (2017).

183. Falagas, M. E., Kastoris, A. C., Karageorgopoulos, D. E. & Rafailidis, P. I. Fosfomycin for the treatment of infections caused by multidrug-resistant non-fermenting Gram-negative bacilli: a systematic review of microbiological, animal and clinical studies. *Int. J. Antimicrob. Agents* **34**, 111–120 (2009).

184. Gutiérrez Martin, C. B. & Rodríguez Ferri, E. F. In vitro susceptibility of Pasteurella multocida subspecies multocida strains isolated from swine to 42 antimicrobial agents. *Zentralbl. Bakteriol.* **279**, 387–393 (1993).

185. Reparaz, J. & Fernández, C. Sensitivity of Vibrio spp. to fosfomycin. *Chemotherapy* **23 Suppl 1**, 58–62 (1977).

186. Rodríguez-Avial, C., Rodríguez-Avial, I., Hernández, E. & Picazo, J. J. [Increasing prevalence of fosfomycin resistance in extended-spectrum-beta-lactamase-producing Escherichia coli urinary isolates (2005-2009-2011)]. *Rev. Esp. Quimioter. Publ. Of. la Soc. Esp. Quimioter.* **26**, 43–46 (2013).

187. Oteo, J. *et al.* CTX-M-15-producing urinary Escherichia coli O25b-ST131-phylogroup B2 has acquired resistance to fosfomycin. *J. Antimicrob. Chemother.* **64**, 712–717 (2009).

188. Oteo, J. *et al.* Parallel increase in community use of fosfomycin and resistance to fosfomycin in extended-spectrum beta-lactamase (ESBL)-producing Escherichia coli. *J. Antimicrob. Chemother.* **65**, 2459–2463 (2010).

189. Betrán, A., Cortés, A. M. & López, C. [Evaluation of antibiotic resistance of Escherichia coli in urinary tract infections in Primary Care Barbastro Sector (Huesca)]. *Rev. Esp. Quimioter. Publ. Of. la Soc. Esp. Quimioter.* **28**, 263–266 (2015).

190. Treviño, M. *et al.* [Surveillance of antimicrobial susceptibility of Escherichia coli producing urinary tract infections in Galicia (Spain)]. *Rev. Esp. Quimioter. Publ. Of. la Soc. Esp. Quimioter.* **29**, 86–90 (2016).

191. Briongos-Figuero, L. S. *et al.* Epidemiology, risk factors and comorbidity for urinary tract infections caused by extended-spectrum beta-lactamase (ESBL)-producing enterobacteria. *Int. J. Clin. Pract.* **66**, 891–896 (2012).

192. Kresken, M. *et al.* Comparative in vitro activity of oral antimicrobial agents against Enterobacteriaceae from patients with community-acquired urinary tract infections in three European countries. *Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis.* **22**, 63.e1-63.e5 (2016).

193. Schmiemann, G., Gágyor, I., Hummers-Pradier, E. & Bleidorn, J. Resistance profiles of urinary

tract infections in general practice--an observational study. *BMC Urol.* **12**, 33 (2012).

194. Kaase, M., Szabados, F., Anders, A. & Gatermann, S. G. Fosfomycin susceptibility in carbapenem-resistant Enterobacteriaceae from Germany. *J. Clin. Microbiol.* **52**, 1893–1897 (2014).

195. Martin, D. *et al.* Prevalence of extended-spectrum beta-lactamase producing Escherichia coli in community-onset urinary tract infections in France in 2013. *J. Infect.* **72**, 201–206 (2016).

196. Meier, S., Weber, R., Zbinden, R., Ruef, C. & Hasse, B. Extended-spectrum β-lactamase-producing Gram-negative pathogens in community-acquired urinary tract infections: an increasing challenge for antimicrobial therapy. *Infection* **39**, 333–340 (2011).

197. Cao, X.-L. *et al.* High prevalence of fosfomycin resistance gene fosA3 in bla CTX-M-harbouring Escherichia coli from urine in a Chinese tertiary hospital during 2010-2014. *Epidemiol. Infect.* **145**, 818–824 (2017).

198. Ho, P. L. *et al.* Dissemination of plasmid-mediated fosfomycin resistance fosA3 among multidrug-resistant Escherichia coli from livestock and other animals. *J. Appl. Microbiol.* **114**, 695–702 (2013).

199. Wheat, P. F. History and development of antimicrobial susceptibility testing methodology. *J. Antimicrob. Chemother.* **48 Suppl 1**, 1–4 (2001).

200. The European Committee on Antimicrobial Susceptibility Testing - EUCAST.

201. Ed., C. P. S. for A. S. T. 31st & 2021, C. supplement M. C. and L. S. I. CLSI. Performance Standards for Antimicrobial Susceptibility Testing. 32nd ed. CLSI supplement M100. Clinical and Laboratory Standards Institute; 2022.

202. Benkova, M., Soukup, O. & Marek, J. Antimicrobial susceptibility testing: currently used methods and devices and the near future in clinical practice. *J. Appl. Microbiol.* **129**, 806–822 (2020).

203. ISO. International Organization for Standarization ISO 20776-1:2020 - Susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices. https://standards.iteh.ai/catalog/standards/cen/e697de2b-10a8-449a-813b-23f9da71c30c/en-iso-20776-1-2020.

204. Brown, D. F. J. & Brown, L. Evaluation of the E test, a novel method of quantifying antimicrobial activity. *J. Antimicrob. Chemother.* **27**, 185–190 (1991).

205. Turnidge, J. & Paterson, D. L. Setting and revising antibacterial susceptibility breakpoints. *Clin. Microbiol. Rev.* **20**, 391–408, table of contents (2007).

206. Martinez, J. L. General principles of antibiotic resistance in bacteria. *Drug Discov. Today. Technol.* **11**, 33–39 (2014).

207. EUCAST. EUCAST: New S, I and R definitions. https://www.eucast.org/newsiandr (2019).

208. Kahlmeter, G. *et al.* European harmonization of MIC breakpoints for antimicrobial susceptibility testing of bacteria. *J. Antimicrob. Chemother.* **52**, 145–148 (2003).

209. EUCAST. MIC distributions and epidemiological cut-off values. https://mic.eucast.org/.

210. EUCAST. EUCAST Antimicrobial wild type distributions of microorganisms. https://mic.eucast.org/search/?search%5Bmethod%5D=diff&search%5Bantibiotic%5D=100&search%5Bspecies%5D=-1&search%5Bdisk_content%5D=-1&search%5Blimit%5D=50.

211. Asín-Prieto, E., Rodríguez-Gascón, A. & Isla, A. Applications of the pharmacokinetic/pharmacodynamic (PK/PD) analysis of antimicrobial agents. *J. Infect.*

Chemother. Off. J. Japan Soc. Chemother. **21**, 319–329 (2015).

212. *EUCAST General Consultation on Fosfomycin IV breakpoints*.

213. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters, version 10.0, 2020.

214. Czechowska, K., Johnson, D. R. & van der Meer, J. R. Use of flow cytometric methods for single-cell analysis in environmental microbiology. *Curr. Opin. Microbiol.* **11**, 205–212 (2008).

215. Afroz, T., Biliouris, K., Kaznessis, Y. & Beisel, C. L. Bacterial sugar utilization gives rise to distinct single-cell behaviours. *Mol. Microbiol.* **93**, 1093–1103 (2014).

216. Lidstrom, M. E. & Konopka, M. C. The role of physiological heterogeneity in microbial population behavior. *Nat. Chem. Biol. 2010 610* **6**, 705–712 (2010).

217. Kærn, M., Elston, T. C., Blake, W. J. & Collins, J. J. Stochasticity in gene expression: from theories to phenotypes. *Nat. Rev. Genet. 2005 66* **6**, 451–464 (2005).

218. Lewis, K. Persister Cells. *https://doi.org/10.1146/annurev.micro.112408.134306* **64**, 357–372 (2010).

219. El-Halfawy, O. M. & Valvano, M. A. Antimicrobial Heteroresistance: an Emerging Field in Need of Clarity. *Clin. Microbiol. Rev.* **28**, 191 (2015).

220. Andersson, D. I., Nicoloff, H. & Hjort, K. Mechanisms and clinical relevance of bacterial heteroresistance. *Nat. Rev. Microbiol.* **17**, 479–496 (2019).

221. Engel, H. *et al.* Heteroresistance to fosfomycin is predominant in Streptococcus pneumoniae and depends on the murA1 gene. *Antimicrob. Agents Chemother.* **57**, 2801–2808 (2013).

222. Walsh, C. C., McIntosh, M. P., Peleg, A. Y., Kirkpatrick, C. M. & Bergen, P. J. In vitro pharmacodynamics of fosfomycin against clinical isolates of Pseudomonas aeruginosa. *J. Antimicrob. Chemother.* **70**, 3042–3050 (2015).

223. Campos, A. C. da C. *et al.* Characterization of fosfomycin heteroresistance among multidrug-resistant Escherichia coli isolates from hospitalized patients in Rio de Janeiro, Brazil. *J. Glob. Antimicrob. Resist.* **22**, 584–593 (2020).

224. Lucas, A. E. *et al.* Frequency and mechanisms of spontaneous fosfomycin nonsusceptibility observed upon disk diffusion testing of Escherichia coli. *J. Clin. Microbiol.* **56**, (2018).

225. Prestinaci, F., Pezzotti, P. & Pantosti, A. Antimicrobial resistance: A global multifaceted phenomenon. *Pathogens and Global Health* vol. 109 309–318 (2015).

226. Docobo-Pérez, F. *et al.* Pharmacodynamics of fosfomycin: Insights into clinical use for antimicrobial resistance. *Antimicrob. Agents Chemother.* **59**, 5602–5610 (2015).

227. Ballestero-Téllez, M. *et al.* Role of inoculum and mutant frequency on fosfomycin MIC discrepancies by agar dilution and broth microdilution methods in Enterobacteriaceae. *Clin. Microbiol. Infect.* **23**, 325–331 (2017).

228. EUCAST. EUCAST: Clinical breakpoints and dosing of antibiotics. Definitions of clinical breakpoints and ECOFF. https://www.eucast.org/clinical_breakpoints.

229. Camarlinghi, G. *et al.* Discrepancies in fosfomycin susceptibility testing of KPC-producing Klebsiella pneumoniae with various commercial methods. *Diagn. Microbiol. Infect. Dis.* **93**, 74–76 (2019).

230. Abbott, I. J. *et al.* Fosfomycin efficacy and emergence of resistance among Enterobacteriaceae in an in vitro dynamic bladder infection model. *J. Antimicrob. Chemother.* **73**, 709–719 (2018).

231. Elliott, Z. S. *et al.* The Role of fosA in challenges with fosfomycin susceptibility testing of multispecies Klebsiella pneumoniae carbapenemase-producing clinical isolates. *J. Clin. Microbiol.* **57**, (2019).

232. Portillo-Calderón, I. *et al.* Contribution of hypermutation to fosfomycin heteroresistance in Escherichia coli. *J. Antimicrob. Chemother.* (2020) doi:10.1093/jac/dkaa131.

233. Agencia española del medicamento y productos sanitarios, A. Ficha técnica: Foscavir 24mg/mL solución para perfusión. (2020).

234. Kim, J.-S. *et al.* CRISPR/Cas9-Mediated Re-Sensitization of Antibiotic-Resistant Escherichia coli Harboring Extended-Spectrum β-Lactamases. *J. Microbiol. Biotechnol.* **26**, 394–401 (2016).

235. Martínez-Gómez, K. *et al.* New insights into Escherichia coli metabolism: carbon scavenging, acetate metabolism and carbon recycling responses during growth on glycerol. *Microb. Cell Fact.* **11**, 46 (2012).

236. Edgar, J. R. & Bell, R. M. Biosynthesis in Escherichia coli of sn-glycerol 3-phosphate, a precursor of phospholipid. Kinetic characterization of wild type and feedback-resistant forms of the biosynthetic sn-glycerol-3-phosphate dehydrogenase. *J. Biol. Chem.* **253**, 6354–6363 (1978).

237. Portillo-Calderón, I. *et al.* Activity of fosfomycin and amikacin against fosfomycin-heteroresistant Escherichia coli strains in a hollow-fiber infection model. *Antimicrob. Agents Chemother.* **65**, (2021).

238. Kahan, F. M., Kahan, J. S., Cassidy, P. J. & Kropp, H. The mechanism of action of fosfomycin (phosphonomycin). *Ann. N. Y. Acad. Sci.* **235**, 364–386 (1974).

239. Martin-Gutiérrez, G. *et al.* Urinary tract conditions affect fosfomycin activity against Escherichia coli strains harboring chromosomal mutations involved in fosfomycin uptake. *Antimicrob. Agents Chemother.* **62**, (2018).

240. Schwöppe, C., Winkler, H. H. & Neuhaus, H. E. Properties of the glucose-6-phosphate transporter from Chlamydia pneumoniae (HPTcp) and the glucose-6-phosphate sensor from Eschetichia coli (UhpC). *J. Bacteriol.* **184**, 2108–2115 (2002).

241. Turner, A. K. *et al.* A genome-wide analysis of Escherichia coli responses to fosfomycin using TraDIS-Xpress reveals novel roles for phosphonate degradation and phosphate transport systems. *J. Antimicrob. Chemother.* **75**, 3144–3151 (2020).

242. Santos-Beneit, F. The Pho regulon: A huge regulatory network in bacteria. *Frontiers in Microbiology* vol. 6 (2015).

243. Abbott, I. J. *et al.* Evaluation of pooled human urine and synthetic alternatives in a dynamic bladder infection in vitro model simulating oral fosfomycin therapy. *J. Microbiol. Methods* **171**, 105861 (2020).

244. Silver, L. L. Fosfomycin: Mechanism and Resistance. *Cold Spring Harb. Perspect. Med.* **7**, (2017).

245. Brook, I., Wexler, H. M. & Goldstein, E. J. C. Antianaerobic antimicrobials: Spectrum and susceptibility testing. *Clin. Microbiol. Rev.* **26**, 526–546 (2013).

246. Swenson, J. M., Killgore, G. E. & Tenover, F. C. Antimicrobial susceptibility testing of Acinetobacter spp. by NCCLS broth microdilution and disk diffusion methods. *J. Clin. Microbiol.* **42**, 5102–5108 (2004).