Department of Microbiology

Faculty of Medicine

University of Seville

Effect of urinary tract physiological conditions on quinolones and fosfomycin activity against *Escherichia coli*

Doctoral Thesis

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Department of Microbiology

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This PhD was performed between March 2014 to December 2017 in the research group of Bacterial Stress and Evolution at the Biomedicine Research Institute of Seville (IBiS). The results obtained during these years are product of scientific work carried out in cooperation with the University Hospital Virgen del Rocío and Virgen Macarena of Seville and the Righospitalet of Copenhagen.

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The Thesis entitled "*Effect of urinary tract physiological conditions on quinolones and fosfomycin activity against Escherichia coli*", submitted by Guillermo Martín Gutiérrez for the degree of Doctor in Biomedical Research (Department of Microbiology, Faculty of Medicine, University of Seville), meets the requirements to opt for the ***International Doctor*** mention.

For all intents and purposes, the following certification is signed:

Jesús Blázquez Gómez	Jerónimo Rodríguez Beltrán	Javier Aznar Martín

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Resumen

Las infecciones del trato urinario (ITUs) constituyen una de las infecciones bacterianas más frecuentes tanto a nivel comunitario como hospitalario. Durante los últimos años se ha descrito un aumento en las tasas de resistencia a los antibióticos así como de las tasas de recurrencias en pacientes con ITU, teniendo un alto impacto tanto en la morbilidad como en la mortalidad en todo el mundo. Para poder hace frente a este problema debemos entender los procesos evolutivos que dan lugar al desarrollo de resistencias durante el tratamiento de las ITUs. Por ello, el objetivo de esta tesis ha sido evaluar el impacto de las condiciones fisiológicas del tracto urinario sobre la actividad de ciprofloxacino y fosfomicina frente a *Escherichia coli.*

En la primera parte de esta tesis hemos evaluado el efecto de las condiciones fisiológicas del trato urinario sobre la actividad de ciprofloxacino sobre cepas con las mutaciones que confieren bajo nivel de resistencia a quinolonas más comunes. Todas las cepas que portaban mecanismos cromosómicos y plasmídicos de bajo nivel de resistencia se convertían en resistentes a ciprofloxacino bajo las citadas condiciones. Nuestros datos sugieren que los métodos recomendados para el estudio de sensibilidad parecen ser un estimador pobre de la actividad de ciprofloxacino frente a cepas de *E. coli* con mecanismos de bajo nivel de resistencia en ITUs, sobreestimando su actividad, particularmente en pacientes con orina con pH ácido.

En la última sección, hemos evaluado el efecto de las condiciones fisiológicas del tracto urinario sobre la actividad de fosfomicina. Para ello utilizamos cepas de *E. coli* portadoras de distintas mutaciones en genes que confieren resistencia a fosfomicina. Nuestros resultados muestran que los métodos estándar para estudiar la sensibilidad frente a fosfomicina pueden sobreestimar su efecto, principalmente en pacientes con valores de pH en orina elevados.

Summary

Urinary tract infections (UTIs) are among the most common bacterial infections acquired in the community and in the hospital. During the last years increasing rates of antibiotic resistance and high recurrence rates have been described in patients with UTI, which have impacted on morbidity and mortality rates and have increased hospitalization costs worldwide. In order to rise to this challenge we must understand the evolutionary pathways that give rise to resistance during UTI treatments. The goal of this thesis was to evaluate the impact of urinary tract physiological conditions on ciprofloxacin and fosfomycin activity against *Escherichia coli.*

In the first part of this thesis, we have evaluated the impact of the urinary tract physiological conditions on the antimicrobial activity of ciprofloxacin against low-level quinolone-resistant (LLQR) strains carrying the most frequent chromosomal mutations. Our results demonstrate that all the LLQR strains studied became resistant to ciprofloxacin under physiological conditions. Subsequently, we demonstrate that under specific urinary tract physiological conditions, susceptible laboratory and clinical strains harboring *qnr* determinants become fully resistant to ciprofloxacin. Thus, our data strongly suggest that recommended methods for MIC determination produce poor estimations of CIP activity against LLQR *E. coli* in UTIs, overestimating its activity, mainly in patients with acidic urine pH.

In the last section, the effect of physiological UTI conditions on fosfomycin activity was evaluated. With this purpose, we used *E. coli* strains harboring low-level fosfomycin resistance (LLFR) mutations. We demonstrate that standard susceptibility testing in Mueller-Hinton with G6P could overestimate the effect of fosfomycin, mainly in patients with basic urine pH values.

‘*The thoughtless person playing with penicillin treatment is morally responsible for the death of the man who succumbs to infection with the penicillin-resistant organism*’

–Sr. Alexander Fleming, 1945.

Index

Chapter I

List of Publications

Publications derived from this PhD thesis

This doctoral thesis has been written as a compendium of published peer reviewed articles based on specific regulations of the PhD program of the University of Seville.

Peer reviewed scientific publications presented as **Publication I, II** and **III** of this PhD thesis are listed below, whose impact factor and position are detailed according to the last update of the Journal Citation Report (2016):

φ **Guillermo Martín-Gutiérrez**; Jerónimo Rodríguez-Beltrán; José Manuel Rodríguez-Martínez; Coloma Costas; Javier Aznar; Álvaro Pascual; Jesús Blázquez. Urinary tract physiological conditions promote ciprofloxacin resistance in low-level quinolone resistant *Escherichia coli*. ***Antimicrobial Agents and Chemotherapy*** 2016, 20: 60(7):4252-8.

φ **Guillermo Martín-Gutiérrez**; José Manuel Rodríguez-Martínez; Álvaro Pascual; Jerónimo Rodríguez-Beltrán; Jesús Blázquez. Plasmidic *qnr* Genes Confer Clinical Resistance to Ciprofloxacin under Urinary Tract Physiological Conditions. ***Antimicrobial Agents and Chemotherapy*** 2017, 24: 61(4): pii: e02615-16.

φ **Guillermo Martín-Gutiérrez**, Fernando Docobo-Pérez, Jerónimo Rodriguez-Beltrán, José Manuel Rodríguez-Martínez, Javier Aznar, Álvaro Pascual, Jesús Blázquez. Urinary tract conditions affect fosfomycin activity *against Escherichia coli* strains harboring chromosomal mutations involved in fosfomycin uptake. ***Antimicrobial Agents and Chemotherapy*** 2017, 21: 62(1): pii: e01899-17

Impact Factor of *Antimicrobial Agents and Chemotherapy* journal: 4.302.
Rank 24/125 in the category Microbiology (Q1).

Chapter II

Abbreviations

Abbreviations:

cAMP: cyclic AMP.

CIP: Ciprofloxacin.

CMQR: Chromosomal-Mediated Quinolone Resistance.

CRP: cAMP receptor protein

DDD: Defined Daily Dose.

DNA: Deoxyribonucleic Acid.

MIC: Minimal Inhibitory Concentration.

FOS: Fosfomycin.

G6P: Glucose-6-phosphate.

IBC: Intracellular Bacterial Communities.

LLFR: Low-Level Fosfomycin Resistance.

LLQR: Low-Level Quinolone Resistance.

LLR: Low-Level Resistance.

PMQR: Plasmidic-Mediated Quinolone Resistance.

ROS: Reactive Oxygen Species.

UPEC: Uropathogenic *Escherichia coli*.

UTI: Urinary Tract Infection

Chapter III

Introduction

3.1. Urinary Tract Infection

Urinary tract infections (UTIs) are among the most common bacterial infections in humans, representing the second most frequent community-acquired infection in women [1,2]. It is estimated that 50% of women have at least one symptomatic UTI episode during their lifetime, and between 20-30% suffer recurrent episodes [2]. Studies suggest that up to 95% of all UTIs are due to an ascending route of infection [3]. Women are more susceptible to UTI than men because their urethral opening is close to the vaginal cavity and rectum, facilitating the movement of bacteria from these sites into the urethra. [4]. Furthermore, the shorter distance from the urethral meatus to the bladder makes it easier for bacterial to reach the bladder by progressive ascending colonization [5]. Although a higher prevalence of UTI is associated with young women, UTI epidemiology depends on the period of life. UTIs occur more often in male than female neonates, whereas an almost equal proportion during infancy and young childhood is found [6]. Regarding elderly population, the frequency of male UTI increases with age, probably secondary as a side-effect associated with voiding problems [7].

UTIs are classified as either lower (confined to the bladder) or upper (pyelonephritis), and as either uncomplicated or complicated [4]. Uncomplicated UTIs affect healthy individuals without structural or neurological urinary tract abnormalities [8]. Complicated UTIs are defined as UTIs associated with factors that compromise the urinary tract or the immune status of patients: pregnancy, urinary obstruction; urinary retention; presence of foreign bodies (catheters or drainage devices); renal failure; renal transplantation or immunosuppression [9]. This distinction has been used to guide the selection and duration of antimicrobial treatments, with

broader-spectrum agents and longer courses often recommended for patients with complicated UTIs [5].

Regarding the etiology of UTIs, the bacteriology is very predictable: although a high number of species can cause UTIs in humans, the majority of infections in all populations are caused by the Gram-negative, facultative anaerobic, *Escherichia coli* [4].

3.1.1. Uropathogenic *Escherichia coli*

Uropathogenic *Escherichia coli* (UPEC) are a heterogeneous group within the classification of extraintestinal pathogenic *E. coli* strains [10]. These bacteria are the primary cause of community-acquired UTI (>80%) and a large portion of nosocomial urinary infections (>50%), accounting for substantial medical costs and morbidity worldwide [2]. In general, UPEC strains differ from commensal *E. coli* strains in that the former possess additional genetic material, often on pathogenicity associated islands, which code for gene products that may contribute to bacterial pathogenesis [11]. Some of these genes allow UPEC strains to express a battery of virulence factors that are proposed to play a role in their ability to cause disease, including fimbriae, adhesins, toxins, flagella, autotransporter proteins and iron-acquisition systems [12]. The ascension of the urinary tract is mediated by the action of flagella, propelling up UPEC strains from the urethra to the bladder.

Once in the bladder, UPEC strains are able to form intracellular bacterial communities (IBC), which protect them from neutrophils, antibiotics and other stresses [9]. Figure 1 shows the pathogenic cycle of UPEC infection within the bladder. The first step is the attachment of UPEC strains to the urothelium through the binding of UPEC peritrichous filamentous adhesive organelles (known as type 1 pili or fimbriae) to a

variety of mannose-containing glyco-protein receptors in the bladder cells [13]. After adhesion UPEC is initially transferred into membrane-bound compartments that are similar to late endosomes [14]. Then, bacteria replicate and form large biofilm-like communities. During IBC maturation, a subpopulation of UPEC progresses into a distinct developmental phase in which cell septation is inhibited, which leads to the formation of filamentous bacteria [15]. Eventually, the integrity of the infected cells is compromised, and bacteria begin to spill out into urine. The emergent bacteria are often highly motile and infect adjacent cells or, eventually, can be flushed out of the urinary tract with the flow of urine [16].

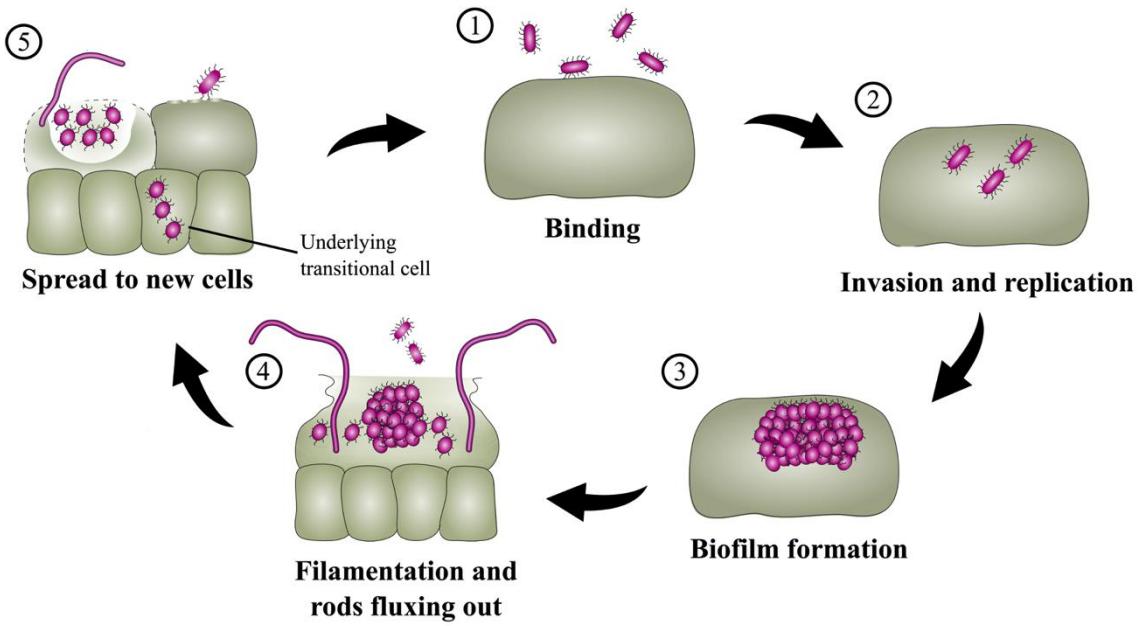

Figure 1. Pathogenic pathway of UPEC during UTI. Figure adapted from [17].

Considering that antibiotic treatment is our primary, and in many cases only, method of treating UTIs caused by UPEC, it is essential to determine the best treatment in order to eradicate the infection, preventing the emergence of antimicrobial resistance and the risk of recurrence.

3.2. UTI treatment and antimicrobial resistance

There is general agreement that all symptomatic UTIs should be treated [1]. In the absence of antibiotic therapy, up to 60% of women experience symptoms and/or bacteriuria after initial infection [18–20], implying that cystitis is not always self-limiting[21]. Furthermore, if the infection persists without adequate treatment, in some cases bacteria ascend through the ureters, causing pyelonephritis and sepsis [22]. For this reason, patients suffering from UTI are commonly treated with antibiotics. In this way, antibiotic therapy should be individualized based on patient circumstances (allergy, tolerability, compliance) [5], spectrum and susceptibility patterns of the aetiological uropathogens, adverse ecological effects, cost and availability [23].

Recently the Infectious Diseases Society of America and the European Society for Microbiology and Infectious Diseases updated clinical practice guidelines for the treatment of uncomplicated cystitis and pyelonephritis [24]. The guideline panel, based in the study published by Peterson DL [25], suggest that the risk of collateral antibiotic damage should be given equal weight as antibiotic efficacy in treatment recommendations. According to this premise, the guideline panel listed four oral antimicrobials for first-line empiric treatment of uncomplicated cystitis: fosfomycin-trometamol (3g p.o. in one dose); nitrofurantoin monohydrate/macrocystals (100 mg twice daily for 5 days); trimetropin-sulfometoxazol (160/800 mg twice daily for 3 days); or pivmecillanam (400 mg orally twice daily for 5 days). As second-line treatments, the guideline recommends ciprofloxacin (250 mg twice daily for 3 days), levofloxacin (250-500 mg once daily for 3 days) and β-lactams (amoxicillin-clavulanate, cefdinir or cefaclor for 3-7 days). Fluoroquinolones are the only oral antimicrobials recommended for empiric treatment of uncomplicated pyelonephritis in

outpatients [24]. Non-hospitalized patients should receive either oral ciprofloxacin (500 mg twice daily for 7 days or 1000 mg extended release daily for 7 days) or levofloxacin (750 mg once daily for 5 days), with this oral treatment preceded by an intravenous antimicrobial treatment such as ceftriaxone or a consolidated 24h dose of an aminoglycoside. Complicated UTIs are associated with a wide range of clinical syndromes and bacteria including multidrug-resistance, making it difficult to generalize regarding treatment choice [26]. Most clinical trials have evaluated 7–14 days of treatment [27], with a 3 day course not sufficient for eradicating infection [28]. Ten to fourteen days of antibiotics are usually recommended for patients with pyelonephritis, bacteraemia, hypotension and other signs of severe sepsis, whereas a 7 days regimen should adequate for lower UTIs [27,29].

It is important to remark that antibiotic use is a key driver of the increase and spread of antibiotic resistance [30,31]. Although there is evidence that antibiotic-resistant bacteria existed prior to clinical use of antibiotics, clinical use of these agents has certainly been coupled with an increase in the emergence of antibiotic-resistant bacteria [32]. When antibiotics are prescribed, the primary aim is to achieve the highest non-toxic antibiotic concentration in the urinary tract in order to obtain the highest bacterial elimination rate, thus preventing the development of resistant strains [33]. Nevertheless, many treatments fall short of this objective due to suboptimal dosing regimens, poor drug distribution or penetration, poor patient compliance with taking medication, or low antibiotic-activity in the urinary tract [33]. Lawrenson and colleagues proved that whichever antibiotic is prescribed to treat UTI, between 12-16% of the patients returned within 28 days for a further course of antibiotic treatment, with women, pregnant patients and patients with diabetes significantly more likely to require additional treatment [34]. Moreover, UPEC strains exhibit a high recurrence rate. More than 68% of

recurring UTIs are caused by the original *E. coli* strain, persisting more than 1 year after the initial infection [35,36]. It is widely thought that recurrences occur through re-ascension and re-inoculation of the bladder lumen by an UPEC strain that has persisted in the periurethral or fecal flora [37].

Clinical situations where bacteria are exposed to low levels of antibiotics are related with acquisition of antibiotic resistance among UPECs. This situation increases the risk of treatment failure due to the use of an ineffective antibiotic, increasing the time from an initial diagnosis to an effective therapy, and thus increasing morbidity by the use of more toxic antibiotics as subsitutes of those that became ineffective [38].

3.2.1. Low-level resistance

Antibiotics have collateral effects on the normal microbiota of the vagina and gastrointestinal tract, altering its composition and generating a selective pressure leading to development of resistant microorganism selection, particularly when the treatment is of long duration [9]. A critical step during antibiotic resistance development is the presence of strains with slightly higher antibiotic resistance than is common for the susceptible population, but still below the clinical breakpoint [39]. These strains are named low-level resistant (LLR), and are considered and intermediary stage in the development of high-resistant strains. LLR appears to facilitate the selections of fully resistant strains, allowing bacterial growth at clinical relevant concentrations. The importance of these strains is that the LLR phenotype may not be detectable by standard susceptibility testing procedures, being classified as “susceptible” although they carry genetic determinants that reduce the susceptibility to antibiotics [39]. Therefore, LLR mutants are arguably the first step on the evolutionary pathway to producing high-level

resistance, and consequently their dissemination and evolution might influence the outcome of antibiotic treatment of UTIs.

3.2.2. Urinary tract physiological conditions and LLR

The antibiotic effectiveness for the treatment of UTI not only depends on the susceptibility of pathogens and the appropriate antibiotic distribution in bladder and kidneys. Physicochemical and pharmacological properties of antibiotics are important factors related to successful therapy. Therefore, even if an UPEC strain was susceptible and the antibiotic concentration in bladder was optimal, there are several factors in the urinary tract that may compromise the efficacy of antimicrobials, contributing to the survival of a significant number of microorganisms under these conditions. This situation may be enhanced by the presence of LLR mutants, which not only may survive under UTI conditions, but also acquire resistance under antibiotic selective pressures (Figure 2).

Urine is a fluctuating and complex fluid composed of over 95% water, plus sodium, ammonia, phosphate, sulfate, urea, creatinine, proteins, and products processed by the kidney and liver [40]. Urine is thought to be a primary defense mechanisms due to its high osmolality, low pH values and the presence of high concentration of urea that can inhibit bacterial growth. However, UPEC strains are able to grow under these conditions throught efficient use of the available resources [11]. Factors associated with urine, including calcium and magnesium ion concentrations, have previously been shown to affect the outcome of antibiotic susceptibility testing results [41]. The urinary pH level also may affect the antibacterial activity of many therapeutic agents used for the treatment of UTIs. The normal range of urinary pH was previously reported to be as variable as 4.5 to 8.5 [42,43]. In the study performed by So and colleagues [44], most of urine

samples collected from patients with UTI had a pH of 6.5 or lower, which means that antibiotics prescribed for UTIs have to be active under acidic conditions in order to optimally eradicate the infection.

Additionally, the bladder environment is mainly anaerobic during UTI. The oxygen partial pressure in the urine of healthy patients is estimated to be as low as 25-80 mmHg [45], which is directly related with renal metabolic state. However, in patients with urinary infections, the urine oxygen concentration is significantly diminished due to the oxygen consumption by the infecting microbes [46]. Bactericidal antibiotics have been shown to kill bacteria (at least in part) by inducing the production of intracellular damaging reactive oxygen species (ROS) [47,48]. Although in a number of recent works the role of ROS in the lethal effect of bactericidal antibiotics has been questioned [49,50], ROS are currently considered a critical factor in antibiotic-mediated killing [51]. A recent study found that killing efficacy of aminoglycosides, fluoroquinolones and β-lactam antibiotics was significantly diminished under anaerobic conditions [47].

It is known how urinary tract physiological conditions (changes on pH values, urine compounds and anaerobiosis) might compromise the optimal activity of different antibiotics. However the knowledge about how UTI conditions could contribute to the therapeutic failure against LLR mutants is limited. We hypothesized that, due to the reduction of antibiotic activity under these conditions, *E. coli* strains could be exposed to sub-inhibitory antibiotic concentrations, which inhibit bacterial growth but do not kill them. Then, a non-lethal selection may results in the emergence of a broader range of mutant variants, most of which will individually have small phenotypic effects and low fitness cost [33,52], giving rise to the presence of high-level resistant microorganism populations which are able to produce a new infection (Figure 2).

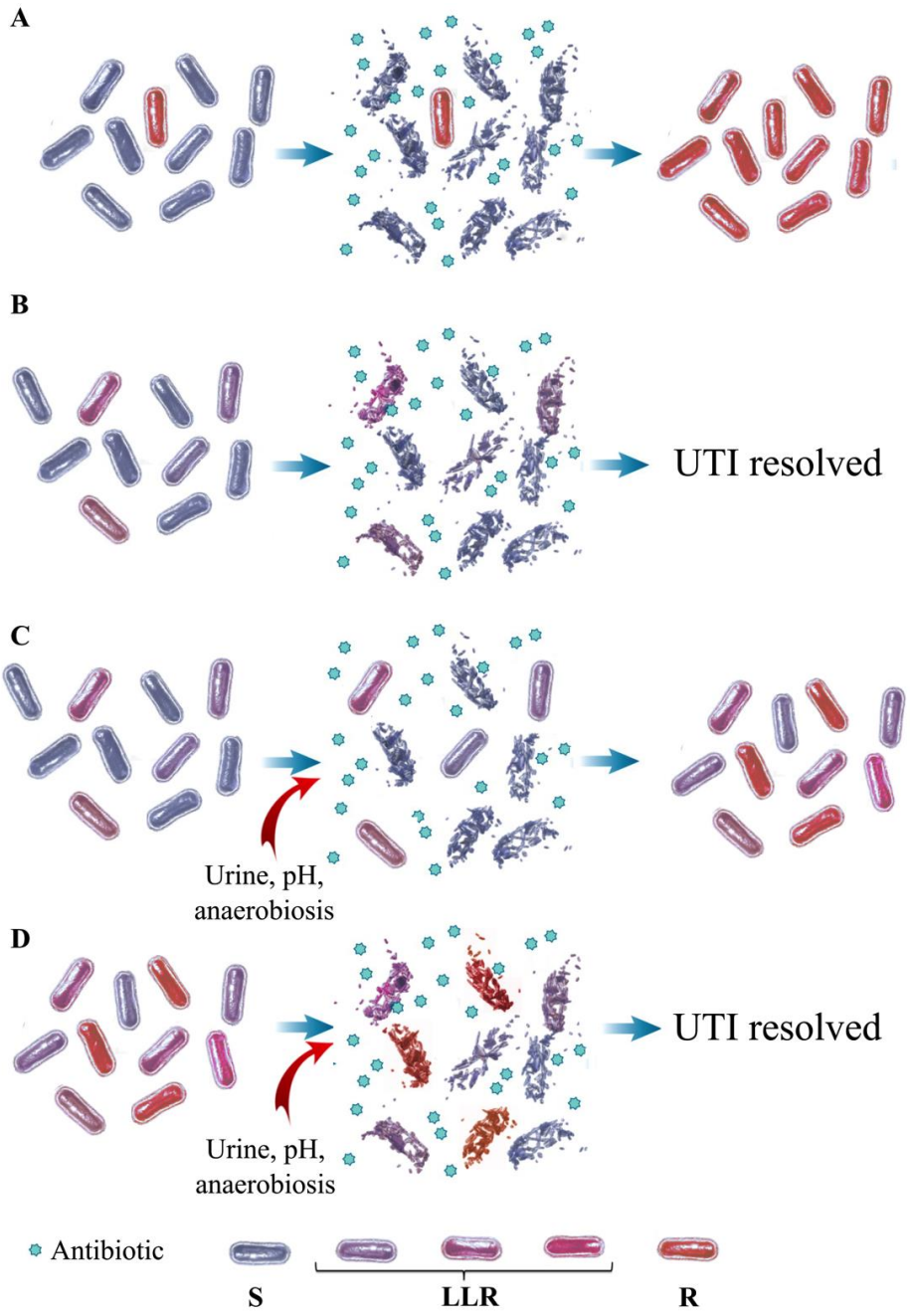

Figure 2. Antibiotic activity and selection for fully resistant (R) and LLR bacteria. **A**) Bacterial population with a subset of resistant organisms. In the presence of an antibiotic, susceptible (S) strains are killed, while resistant strains survive, proliferating and causing a new infection. **B**) Bacterial population with a subset of LLR organisms. In this case, the population is susceptible to the antibiotic, and the infection is resolved. **C**) Bacterial population with a subset of LLR organisms in the presence of UTI conditions. Under these conditions antibiotic activity could be reduced, allowing LLR strains not only to survive but also to acquire resistance under antibiotic selective pressures. **D**) In this case, UTI conditions could increase antibiotic activity, allowing the resolution the infection, even in the presence of R bacteria. Figure adapted from [53].

3.3. Ciprofloxacin treatment in UTIs

Quinolones are molecules structurally derived from the heterobicyclic aromatic compound quinoline, originated from a substance obtained after the alkaline distillation of quinine [54]. The discovery of nalidixic acid in 1962 by Lesher and associates [55] marked the beginning of five decades of quinolone development [56]. Since then, all current quinolone derivatives have a dual ring structure with a nitrogen at position 1, a carbonyl group at position 4, and a carboxyl group attached to the carbon a the 3 position of the firs ring [1] (Figure 3). The potency of quinolones against gram-negative bacteria was significantly improved by the development of fluoroquinolones (with the addition of a 6-fluoro group) extending the therapeutic spectra and enhancing pharmacokinetic properties [57].

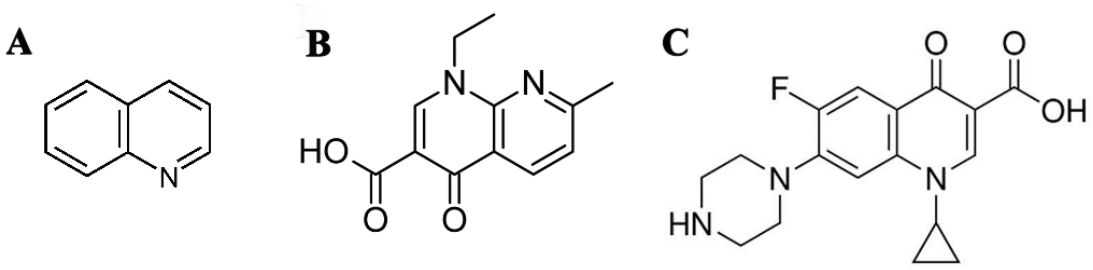

Figure 3. Chemical structures of quinoline (A), nalidixic acid (B), and ciprofloxacin (C).

The fluoroquinolone ciprofloxacin is one of most commonly used agents for UTI treatment [58–60]. It has been employed as an appropriate therapy in patients with UTI not requiring hospitalization in areas where the prevalence of resistance is under 10%. In addition, it is considered an effective treatment in the prevention of UTI in kidney transplant recipients [24,61]. Furthermore, ciprofloxacin shows an excellent bioavailability (over the 70% of the administered dose) [62–64], particularly due to the high absorption, localized preferentially at the duodeno and jejunal level, where it is usually absorbed by

passive diffusion [65,66]. High concentrations of ciprofloxacin (up to 990 mg/L) [67] are reached in urine during oral treatment of UTIs.

Ciprofloxacin is highly effective when given as brief courses of 3-10 days, with efficacy comparable to trimethroprim-sulfamethoxazole [68], nitrofurantoine [69], and better than amoxicillin-clavulanate [70]. In comparison with other fluoroquinolones, ciprofloxacin shows comparable clinical efficacy and bacteriologic eradication rates to levofloxacin [71], ofloxacin [72], sparfloxacin [73] or gatifloxacin [74] for UTI treatments.

3.3.1 Mechanisms of action of fluoroquinolones

Fluoroquinolones inhibit the enzymatic activities of two members of the topoisomerase class of enzymes: DNA gyrase and topoisomerase IV. DNA gyrase, the first-recognized target of quinolones, is composed of two A and two B subunits, products of the *gyrA* and *gyrB* genes, respectively [75]. DNA gyrase catalyzes the introduction of negative superhelical twists into closed covalently circular DNA, and is also responsible for removing positive superhelical twists that accumulate ahead of the DNA replication fork [1]. Topoisomerase IV is composed of two subunits encoded by *parC* and *parE*. The main role of topoisomerase IV seems to be associated with decatenating the daughter replicons [76]. Topoisomerases twist and untwist the DNA helix by binding to it and introducing a pair of staggered, single-strand breaks in one segment, through which a second DNA segment is passed [77].

When a fluoroquinolone is present, the complex DNA-topoisomerase is altered into a drug–enzyme–DNA complex known as a ternary complex, in which the topoisomerase is trapped with the bound DNA [78]. Fluoroquinolones bind to DNA gyrase or topoisomerase IV, which is then unable to re-ligate the DNA substrate [79], thereby

converting the enzyme-DNA complex into a poisonous complex [80]. The broken segments of DNA bound to the enzyme are named as "cleaved complexes". If the level of topoisomerase-mediated DNA cleavage becomes too high, the action of DNA tracking systems can convert these transient complexes to permanent double-stranded breaks [81]. They interact with DNA and the GyrA subunit of the DNA gyrase, or the ParC subunit of the topoisomerase IV, stabilizing the DNA-enzyme cleavage complex, and leading to the blockage of the replication machinery progression, DNA lesions formation, and finally to bacterial death [82].

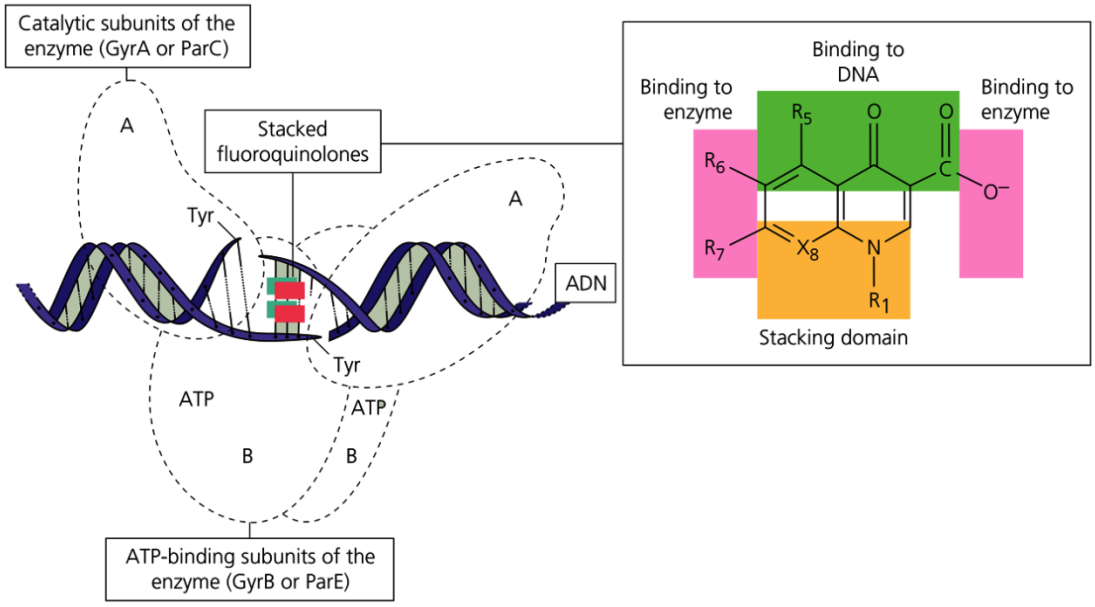

Figure 4. Ternary complex formed between DNA, DNA–gyrase/topoisomerase IV -and stacked fluoroquinolone. The binding site for fluoroquinolones is located in the bubble formed during the local opening of the DNA molecule. The right panel shows the sites of the antibiotic molecules which interact with DNA, with the enzyme, or favoring the stacking of the fluoroquinolone molecules. Adapted from Bambeke *et al.* [83].

3.3.2. Fluoroquinolone resistance in *E. coli*

During recent years, an increase in resistance to fluoroquinolones in *E. coli* strains has been described worldwide [84]. As mentioned above, the prevalence of antibiotic resistant strains has been related with antibiotic use [85]. The European Centre for Disease Prevention and Control (ECDC) provides information about antibiotic consumption and the rates of resistance in *E. coli* strains from all the countries of the European Union. Figure 5 illustrates fluoroquinolone consumption in defined daily dose (DDD) per 1000 patients per day and rates of resistance to fluoroquinolones in invasive *E. coli* strains from 2001 to 2015 in Spain. It can be observed that the progressive increase of resistant *E. coli* strains appears to be related with antibiotic consumption, reaching resistance rates over 34%.

Furthermore, several clinical factors have been considered as possible predictors of fluoroquinolone resistance. Colodner and colleagues found that previous invasive procedures (like urine catheters, foreign bodies, orthopedic devices, etc.), recurrent UTI and previous hospitalization were independent risk factors for community-acquired UTI caused by quinolone resistant *E. coli* strains [86]. The presence of complicated UTI [87,88], urinary catheterization in the previous 6 months [59,89] and male gender [90] have been identified as predictor factors of quinolone resistance in UTI. Increasing age was also associated with an increased risk of UTI by quinolone resistant UPEC, with relatively low resistance rates among UPEC isolates from pediatric outpatients (5.1%), higher among isolates from adult outpatients (11.8%), and the highest among isolates from older adult outpatients (29.1%) [91].

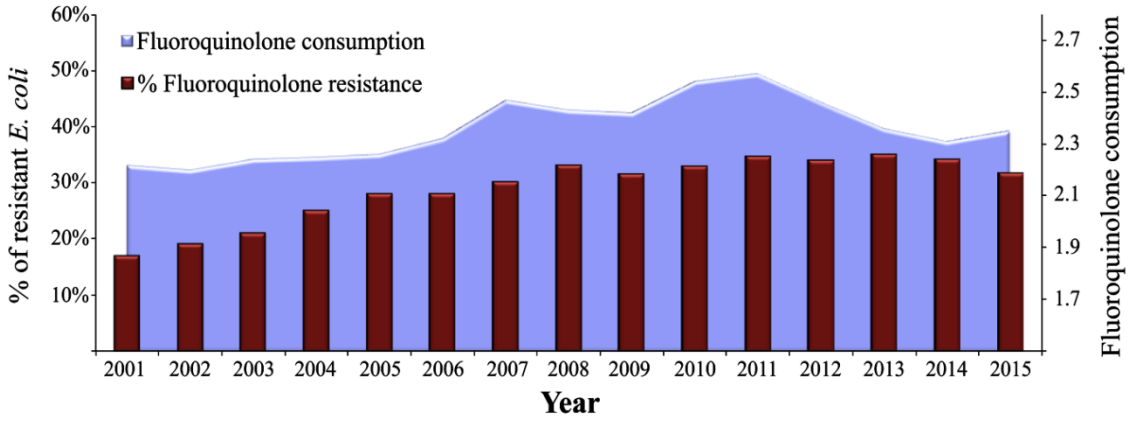

Figure 5. Fluoroquinolone usage and resistance rates in Spain from 2001 to 2015. This graph represents percentage of fluoroquinolone resistant *Escherichia coli* isolates (red bars) and fluoroquinolone consumption in defined daily dose (DDD) per 1000 patients per day (blue area chart) in the community. Data from http://ecdc.europa.eu

3.3.3. Fluoroquinolone resistance mechanisms in *E. coli*

3.3.3.1. Chromosomal-mediated quinolone-resistance

Quinolone resistance is principally acquired through mutations in the genes encoding gyrase and topoisomerase [92]. Target alterations due to mutations in the *gyrA*, *gyrB*, *parC*, and *parE* genes have been described in UPEC, most of which are located in small regions of either *gyrA* or *parC*, known as the quinolone resistance-determining region [93,94]. These chromosomal mediated quinolone-resistance (CMQR) mutations are commonly situated in the amino terminal domains of GyrA (residues 67 to 106 for *E. coli* numbering) or ParC (residues 63–102) [95]. The most common mutation observed in clinical isolates is at codon 83 (S83L) in *gyrA*, followed by codon 87 (D87 to N, Y, G, or H) [96–98]. In ParC, the common substitutions appear to be at codons 80 and/or 84 [97]. Mutations in specific domains of GyrB and ParE have also been shown to cause quinolone resistance, although they are substantially less common in resistant UPEC isolates. The study performed by Lindgren and colleagues [98] found mutations in *parE*

gene in 5 from 54 strains (9.25%), four of them accompanied with mutations in *gyrA* and *parC*. Similar results were obtained by Takahashi and colleagues [99], where 18 from 178 strains (10.11%) presented mutations in the *parE* gene.

3.3.3.2. Efflux pumps and porins

Efflux pumps in *E. coli* have a broad substrate range and transport antibiotics out of the bacterium, conferring intrinsic multidrug resistance [100]. Genes encoding these efflux pumps are classified into the following five superfamilies: the major facilitator superfamily (MFS), small multidrug resistance (SMR), multidrug and toxic compound extrusion (MATE), ATP-binding cassette (ABC), and resistance-nodulation-cell division (RND) [101]. Among RND transporters of *E. coli*, only the AcrB-AcrA-TolC complex (also called AcrBA-TolC) is constitutively expressed and plays a major role in multidrug resistance. It is composed of the inner membrane RND antiporter AcrB that functions in a tripartite assembly with a periplasmic adaptor protein, AcrA, and the outer membrane channel, TolC [100,102]. Inactivation of AcrB increases the susceptibility of laboratory mutants of *E. coli* and other *Enterobacteriaceae* to many antimicrobials, whereas over-expression confers resistance to multiple drugs, including ciprofloxacin [103]. Moreover, mutations in *acrR* (a repressor of acrAB) increase pump activity [104]. The MarR protein, which regulates expression of *marA*, is expressed from the MAR (from multiple antibiotic resistance) operon, regulating the transcription of *acrAB* and *tolC* genes [105]. Thus, inactivation of the repressor MarR increases the expression of *marA*, which amplifies the transcription of the pump genes *acrAB* and *tolC*, increasing the efflux of ciprofloxacin [106].

Three main porins are located in the outer membrane of *E. coli*: OmpA, OmpC and OmpF [76]. Alterations in membrane permeability are usually associated with decreased expression of these porins [107]. Both OmpF and OmpC channels are homotrimers, and in each monomer 16 β-strands span the outer membrane to form a barrel [108]. Genes enconding OmpF and OmpC (*ompF* and *ompC*) are transcriptionally regulated, depending on the temperature and the osmolarity of the media, by the two-component regulatory system OmpR-EnvZ that mediates both positive and negative control [109]. There is also a post-transcriptional control by the small regulatory RNA molecules, *micC* and *micF*, which downregulate OmpC and OmpF expression respectively [110,111]. Furthermore, MarA induce upregulation of an antisense *micF*, causing a significant reduction in expression of *omp*F [109]. The porine OmpF facilitates the diffusion of fluorinated quinolones such as ciprofloxacin and cephalosporins into the periplasm, with marked reductions in accumulation of these agents in the OmpF-deficient bacterial strains. In this way, down-regulation of these porins as well as point mutations can lead to reduced accumulation of quinolones (and other agents) within the bacterial cell [112]. The OmpC channel is smaller than that of OmpF, and for this reason antibiotic molecules with large side chains are more restricted in their permeation through OmpC than through the wider OmpF channel [113].

3.3.3.3. Plasmid-mediated quinolone-resistance

Recently several plasmid-mediated quinolone-resistance (PMQR) mechanisms have been identified. The first report of a plasmid-mediated quinolone resistance describes the presence of a 218 aminoacid protein termed Qnr, that protects DNA from quinolone binding to topoisomerases [114]. Since then, five groups of plasmidic Qnr determinants (encoded by the genes *qnrA, qnrB, qnrC, qnrD and qnrS*) have been

described [115]. The *qnrVC* gene from *Vibrio cholerae* can also be located in a plasmid [116,117] or in transmissible form as part of an integrating conjugative element [118]. These *qnr* genes generally differ in sequence by 35% or more from *qnrA* and each other [119]. Allelic variants have also been described in each family, differing by 10% or less: 5 alleles for *qnrVC*, 7 alleles for *qnrA*, 9 for *qnrS*, and 71 for *qnrB* [120]. Qnr proteins appear to bind to gyrase and topoisomerase IV targets in such a way as to destabilize the cleavage complex enzyme-DNA-quinolone, beginning quinolone release, religation of DNA, and regeneration of active topoisomerase. Moreover, QnrA, QnrB and QnrS have been shown to protect DNA gyrase from quinolones in *E. coli* [80,121–123]. QnrA also protects topoisomerase IV [80]. Furthermore, *qnr* genes are often associated with extended-spectrum β-lactamases and aminoglycoside resistance encoding genes on the same plasmid [124], increasing mutually the probability of dissemination [125].

Additionally, three further PMQR genes have been found: i) *aac(6')-Ib-cr* is a variant aminoglycoside acetyltransferase that reduces ciprofloxacin activity [126]. This enzyme, in addition to aminoglycoside antibiotics, confers resistance to ciprofloxacin and norfloxacin by N-acetylation of the amino nitrogen on its piperazinyl substituent [127,128]; ii) *qepA and oqxAB* encoding efflux pump [129,130]. QepA is a plasmid-mediated efflux pump of the major facilitator family that decreases susceptibility to hydrophilic fluoroquinolones, especially ciprofloxacin, norfloxacin and veterinary enrofloxacin [129]. Concerning *oqxAB*, it is an efflux pump that was initially recognized on transmissible plasmids responsible for resistance to olaquindox [130,131]. It has a wide substrate specificity including chloramphenicol, trimethoprim, and quinolones such as ciprofloxacin, norfloxacin, and nalidixic acid [132].

3.3.3.4. Low-level quinolone-resistance

Resistance to fluoroquinolones is a step-by-step phenomenon involving accumulation of resistance mechanisms. The presence of single chromosomal mutations in *gyrA* or *parC*, as well as the presence of PMQR genes, lead to generation of low-level quinolone resistant (LLQR) strains, with fluoroquinolone MICs that are higher than the epidemiological cutoff value but still below the resistance breakpoint for most fluoroquinolones. A high prevalence of LLQR UPEC mutants in UTIs has been described. Takahashi and colleagues analyzed CMQR genes in 89 fluoroquinolone-susceptible UPEC strains from patients with complicated or uncomplicated cystitis, finding that 16 (17.97%) of these strains were LLQR mutants [99]. Lindgren and colleagues studied 53 strains with susceptibilities that covered the full range of MICs and that were isolated from patients with uncomplicated UTIs, concluding that at least 17 strains (70.91%) from fluoroquinolone-susceptible strains presented CMQR mutations [98]. Additionally, a high prevalence of *qnr* genes has been found in clinical urinary isolates (from 10.81% to 31.6%)[133–135]. This prevalence varies depending on geographical location, patient characteristics (e.g. higher prevalence in inpatients than outpatients) [133] or associated antibiotic susceptibility patterns [135,136].

It should be noted that the causes of this high prevalence of LLQR among UTI isolates remain puzzling: these mutations and PMQR determinants only lead to a slight increase in resistance (below the clinical breakpoint) that should not be enough to survive under the extremely high quinolone concentrations attained in the urinary tract.

3.3.3.5. Effect of urinary tract physiological conditions on ciprofloxacin activity

The effectiveness of ciprofloxacin for the treatment of UTI does not only depend on pathogen susceptibility. It has been emphasized that physicochemical proprieties of quinolones have major consequences for their pharmacokinetics and pharmacodynamics [41]. Ciprofloxacin is an ampholyte that can exist in four different pH-dependent protonation forms namely cation ($H2X^+$), zwitterion ($HX^{\pm}$), neutral (HX^0), and anion (X^-) depending on the pH of the medium [137]. At the isoelectric point of quinolones in water (about pH 7), the mole fractions of the zwitterionic and neutral species reach their maximum values, being zwitterions the most stable species [138]. Figure 6 represents ciprofloxacin speciation as cation, zwitterion, and/or anion as a function of pH values. Antibiotics in zwitterionic forms are optimal for porin permeability, favoring increased bacterial accumulation [139,140].

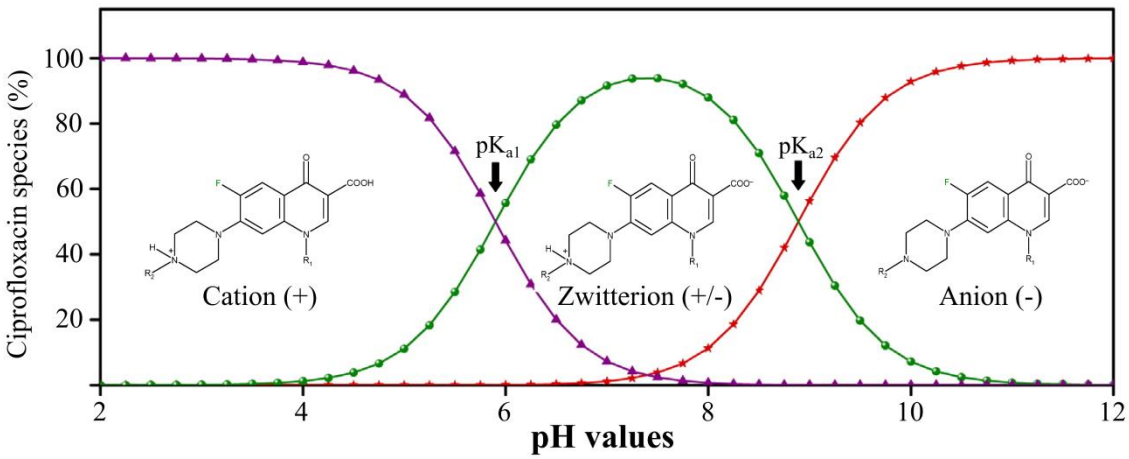

Figure 6. Distribution of ciprofloxacin species as a function of pH. The pKa value given for the carboxyl (pK_{a1}) or amino (pK_{a2}) group refers to the equilibrium between the protonated positive and deprotonated neutral group. Figure adapted from [141].

However, as previously mentioned, most patients with UTI caused by *E. coli* present urinary pH values lower than 6.5. At these pH values ciprofloxacin is cationic (positively charged), decreasing the penetration into bacteria, and thus, reducing its activity [112,142].

In addition to the effect of pH on the activity of ciprofloxacin, there are several urine compounds that can interact with ciprofloxacin, reducing its activity. For instance, divalent urinary cations may also decrease ciprofloxacin activity [143]. In this way, the cation magnesium (Mg^{2+}) interacts with the quinolone carboxyl (COO-) group [144], generating quinolone-magnesium complexes that are significantly less active than the quinolone drugs alone. It has been also found that the activity of quinolones is reduced when the solutions are titrated with magnesium ions [145]. Moreover calcium have also been related with impairment of uptake quinolones by bacteria [146].

Another physiological factor that could reduce quinolone susceptibility is the absence of oxygen under urinary tract conditions. Higher concentrations of ciprofloxacin are needed to kill cells under anaerobic conditions, an observation supported by the higher minimal inhibitory concentration (MIC) values found under this condition [147]. Previous works have shown that fluoroquinolones induce physiological alterations in the cellular redox state, promoting the formation of reactive species including ROS [47,48]. Under anaerobic conditions, ciprofloxacin activity is significantly reduced, but not completely eliminated [47], supporting that the availability of molecular oxygen plays a significant role for the lethality of quinolones [148].

Despite the fact that the effect of pH, urine and anaerobiosis on ciprofloxacin activity has received considerable attention, little is known about how the urinary physiological conditions impact ciprofloxacin activity against LLQR mutants. Under

these conditions, it is possible that LLQR mutants may be as effective as high-level resistant strains, allowing these mutants to survive the clinical concentrations of ciprofloxacin in urine. In addition, it has been demonstrated that fluoroquinolones increase mutagenesis in bacteria via the induction of SOS error-prone DNA polymerases expression, DNA recombination [149] and the horizontal transfer of DNA sequences [150–152]. Thus, the effect of UTI conditions on ciprofloxacin activity, together with the mutagenesis induction produced by this antibiotic, could give place to ITU recurrence by UPEC with a development of a wide spectrum of resistance mechanisms.

3.4. Fosfomycin treatment in UTIs

Fosfomycin is a phosphonic acid derivative produced by a broad variety of *Streptomyces* and *Pseudomonas* species [153]. This antibiotic contains an epoxide and a propyl group (cis-1,2-epoxypropyl phosphoric acid; $C_3H_7PO_4$), with a unique chemical structure (Figure 7) and a very low molecular weight [154]. Moreover, it is unrelated to any other antibiotic family, being a class of its own [153].

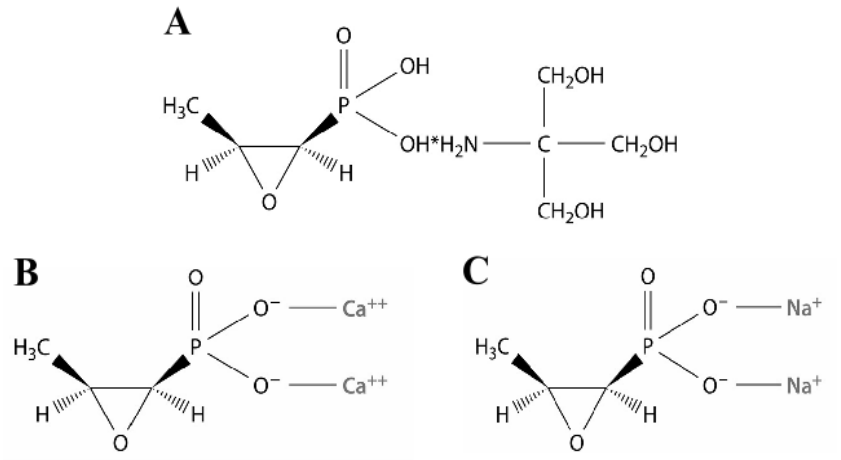

Figure7. Molecular structure of **A**) Fosfomycin trometamol;

B) Fosfomycin calcium; and **C**) Fosfomycin Disodium

Fosfomycin was initially developed in Europe by the Compañia Española de Penicilina y Antibióticos (CEPA) [155], and has been used since the early 1970s, initially as an intravenous preparation of the disodium salt, and later as an oral formulation of fosfomycin trometamol [156]. Additionally, other formulations like fosfomycin calcium for oral use, as well as fosfomycin disodium for intravenous use are also available [157].

Since fosfomycin was discovered in 1969 [155], this natural antibiotic has attracted considerable clinical and scientific interest due to its broad spectrum bactericidal activity against Gram-positive and Gram-negative bacteria [20,158,159]. Furthermore, owing to the emergence of multi-drug resistant bacteria as well as the limited options of new antibiotic agents, fosfomycin is being reevaluated as a potential therapeutic option, being present in numerous clinical guidelines and trials for the treatment of different infections [160–163].

Fosfomycin has been widely used as a first-line agent for the empirical treatment of UTIs [154]. Fosfomycin trometamol is the preferred formulation for oral administration because it is more readily absorbed compared with other formulations. Moreover, it is administered in a single dose, improving compliance, reducing adverse events or toxicity, and reducing the effects on the normal microbiota of the vagina and gastrointestinal tract [164]. It is marketed under the brand name Monuril®, Monurol® or Monural®, at a dose of 5.61 g of fosfomycin trometamol, which is equivalent to approximately 3 g of pure fosfomycin [157]. After oral 3-g dose administration, peak urine concentrations are reached within 4h, where it is possible to find concentrations of 1053-4415 μg/ml [165,166]. Furthermore, after this treatment, high fosfomycin concentrations (>128 μg/ml) persist during 1-2 days in urine and bladder, enough to eradicate any susceptible bacteria [166]. Regarding the efficacy of fosfomycin versus other

antibiotics, the meta-analysis of randomized controlled trials performed by Falagas and colleagues [20] demonstrated that fosfomycin treatment in patients with cystitis was equal to the comparator regimens (quinolones, trimethoprim, trimethoprim-sulfamethoxazole, β-lactams or nitrofurantoin) in terms of clinical effectiveness and safety profile.

3.4.1 Mechanisms of action of fosfomycin

Fosfomycin is a bactericidal antibiotic agent that inhibits an enzyme-catalyzed reaction in the first step of the synthesis of the bacterial cell wall [167]. In *E. coli* fosfomycin is actively transported into bacterial cytoplasm via GlpT and UhpT transporters [168]. The GlpT antiporter is a member of the MFS, which is commonly responsible for the import of glycerol-3-phosphate driven by inorganic phosphate gradient [169]. The UhpT is a chemiosmotic transporter that catalyzes the accumulation of glucose-6-phosphate (G6P) by exchange with internal inorganic phosphate [170]. UhpT is also a member of the MFS that requires transcriptional activation by the response regulators UhpA and UhpB, which together form a two-component regulatory system that activates expression of the transporter UhpT [171]. This activation is produced after recognition of extracellular G6P by the constitutively expressed sensor UhpC, which interacts with UhpB stimulating its kinase activity [172]. After that, a phosphate group is transferred to UhpA, which acts as a transcriptional activator for the expression of the *uhpT* gene. The expression of both GlpT and UhpT transporters are also regulated by cyclic AMP (cAMP) levels in the cell [173].

In *E. coli*, the expression of the *glpT* and *uhpT* genes are activated by the complex of cAMP and its receptor termed cAMP receptor protein (CRP) [174,175], also known as catabolite activator protein (CAP). cAMP synthesis depends on the activity of adenyl cyclase CyaA, which is also regulated by the phosphotransferase enzyme PtsI, a

component of the phosphoenol-pyruvate sugar phosphotransferase transport system [153]. CRP is one of the main global transcription regulators in *E. coli*, whose activity is triggered by binding of cAMP in response to glucose starvation and other stresses [176]. Additionally, the FNR (fumarate-nitrate reduction) protein is a transcriptional regulator containing a Fe-S cluster, serving as a redox sensor, and is active during anaerobic growth [177]. Under this condition, FNR proteins bind to the regions upstream of *glpT* and *uhpT*, acting as an activator of these genes [178]. The regulation of *glpT* and *uhpT* genes is represented in Figure 8.

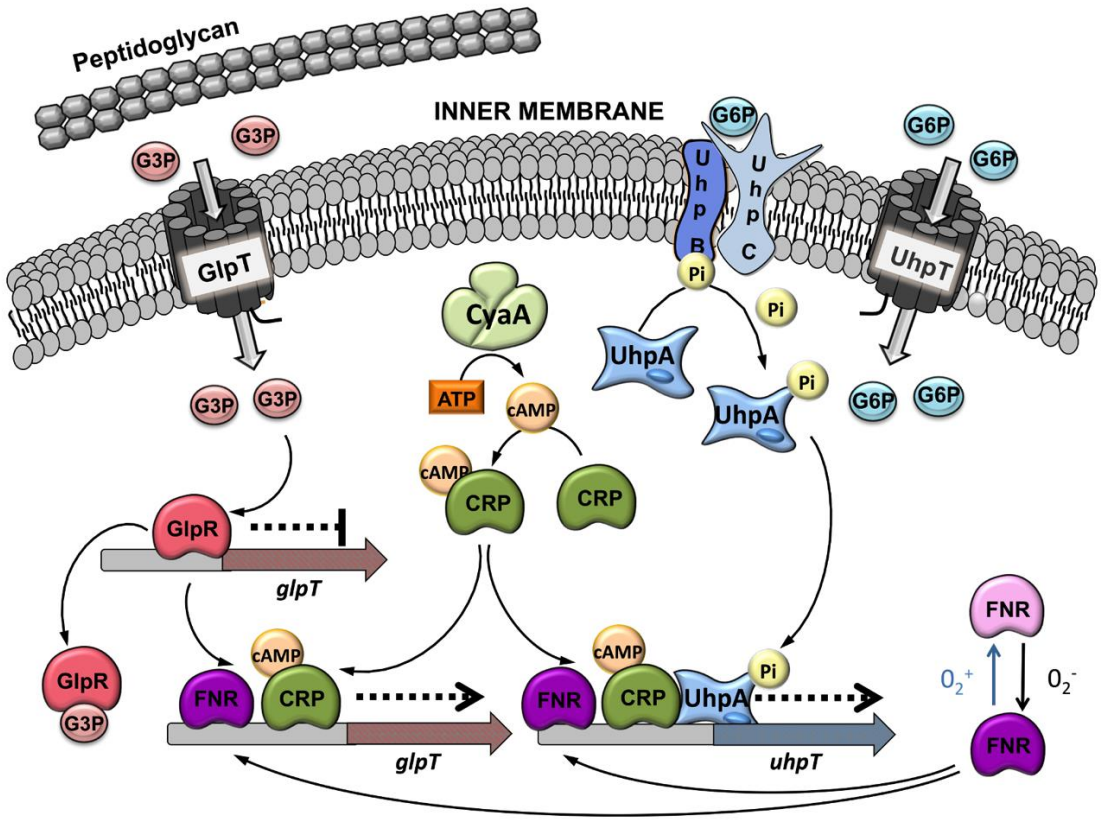

Figure 8. Regulation of *uhpT* and *glpT* gene expression. Figure adapted from [153].

Once has reached the cytoplasm, fosfomycin acts as a phosphoenolpyruvate (PEP) analogue, binding covalently to the thiol group of the Cys115 in the active site of MurA (UDP-GlcNAc enolpyruvyl transferase), an essential enzyme for peptidoglycan biosynthesis [168,179].

Nucleophilic attack on the β-carbon of fosfomycin by Cys115 opens the epoxide to form an irreversible active site modification that eliminates MurA activity [173]. Thus, fosfomycin disrupts the formation of Enolpyruvate-UDP-N-acetylglucosamine acid from UDP-N-acetylglucosamine and phosphoenolpyruvate, which is the initial step in peptidoglycan chain formation of the bacterial wall [168].

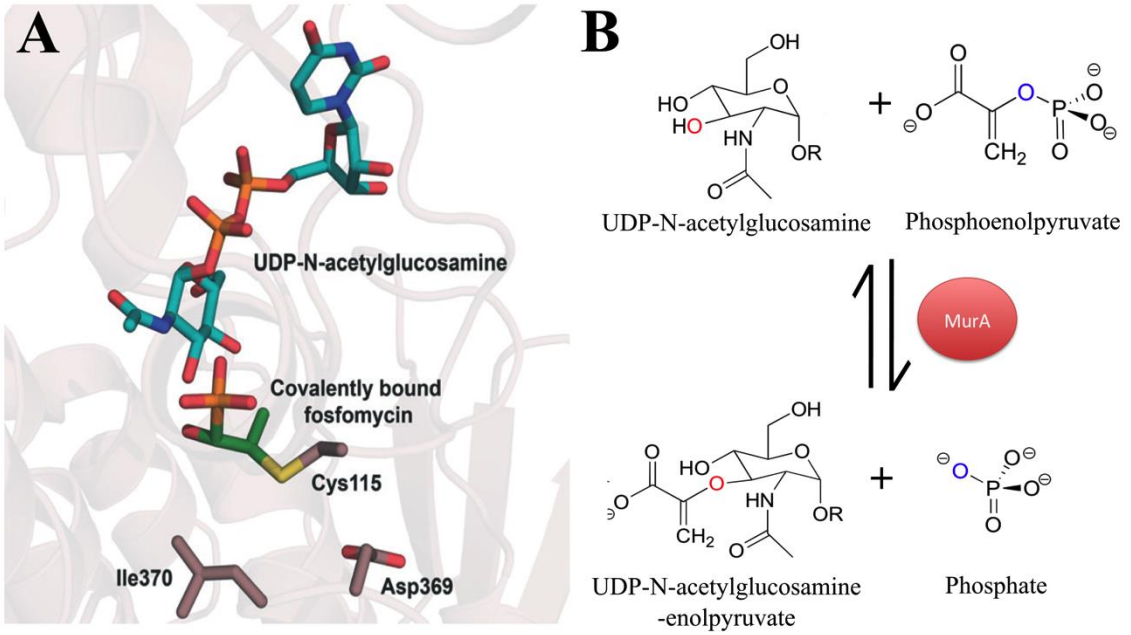

Figure 9. A) Fosfomycin irreversibly modifies MurA by forming a covalent linkage with Cys115. **B**) MurA catalyzes the formation of UDP-N-acetylglucosamine-enolpyruvyl from phosphoenolpyruvate and UDP-N-acetylglucosamine. Figure modified from [173].

3.4.2. Fosfomycin resistance in *E. coli*

The frequency of fosfomycin resistance among UPEC has been recognized to be very low [154,180]. Most of the studies using *E. coli* strains collected from clinical urine samples have revealed high rates of susceptibility, ranging between 95 to 100% [181–185]. The largest of these studies was a multicenter study performed in 20 hospitals in Spain, where 99.8% of 2292 *E. coli* isolates were susceptible to fosfomycin, whereas resistance

rates to other antibiotics like ampicillin, ciprofloxacin and cotrimoxazole were remarkably higher (52.1%, 18.1%, and 25.2% of isolates, respectively) [181].

During recent years, most of the studies published have evaluated the *in vitro* activity of fosfomycin against extended-spectrum β-lactamases (ESBL) producing *Enterobacteriaceae*, particularly in *E. coli* [186–191]. Although the frequency of cross-resistance of fosfomycin and other antibiotics is expected to be very low because of the unique mode of action of this antibiotic [192], Oteo and colleagues showed a parallel increase in the use of fosfomycin in the community and resistance to fosfomycin in ESBL-producing *E. coli* from UTI in Spain [193].

3.4.3. Fosfomycin resistance mechanisms in *E. coli*

3.4.3.1. Chromosomal-mediated fosfomycin-resistance

The most important mechanism of *E. coli* resistance to fosfomycin is the presence of mutations in genes encoding the uptake systems used for fosfomycin entry inside the bacteria [153,154]. Mutations in any of the structural genes of those pathways decrecrease antibiotic uptake, conferring different levels of fosfomycin resistance [168,194]. Strains defective in fosfomycin uptake are not able to grow using some substrates as the only carbon source, such as glycerol-3-phosphate in GlpT-deficient strains or G6P (and other hexose phosphates) in Uhp-deficient strains [183]. Mutants affected in both systems are often unable to grow using multiple carbohydrates. In this way, it has been observed that the addition of G6P induces fosfomycin sensitivity in resistant GlpT-deficient strains, due to the induction of UhpT synthesis [168]. For this reason MIC determinations of fosfomycin are performed with media containing G6P.

As mentioned above, cAMP is required for the full expression of the fosfomycin transporters GlpT and UhpT. Mutations in *cyaA* or *ptsI* produce a decrease in the intracellular cAMP levels and, subsequently, a reduced expression of both fosfomycin transporters, leading to diminished antibiotic uptake [153,183]. Furthermore, inactivation of the cAMP receptor protein CRP affects the expression of both transport systems, decreasing the susceptibility to fosfomycin. cAMP-CRP recognizes several binding sites upstream of the *glpTQ* operon [195,196], upregulating the expression of *glpT*. The complex cAMP-CRP also binds to the *uhpT* promoter at a single site upstream of the UhpA-binding sites, stabilizing the open promoter complexes for *uhpT* transcription, increasing the rate of their formation [175,197]. Although the role of CRP in *glpT* and *uhpT* expression is well known, there is currently a lack of information about how mutations in the *crp* gene could affect fosfomycin susceptibility in *E. coli* clinical isolates.

Fosfomycin resistance by target modification has also been described. Kim and colleagues [198] demonstrated that substitution of Cys 115 by Asp (C115D) retain MurA activity and confer high-resistance to fosfomycin. However, few reports of clinical isolates shown mutations in the *murA* gene, and none in the catalytic site of MurA, because most of them reduce drastically bacterial cell viability [199]. However, overexpression of the *murA* gene in *E. coli* have been correlated with higher levels of fosfomycin resistance, reaching clinical resistance levels at a low fitness cost [200].

3.4.3.2. Plasmid-mediated fosfomycin-resistance

The transfer of a fosfomycin resistance marker encoded by a plasmid by conjugation was described in 1980 in *Serratia marcesens* [201]. Since then, several enzymes able to modify fosfomycin have been described, producing chemical changes that inactivate it. Microbial resistance to fosfomycin by antibiotic modification

transmited by plamids involves five different fosfomycin resistance proteins: FosA [202], FosB [203], FosC [204], FosK [205] and FosX [206].

FosA (glutathione S-transferase) is a metalloenzyme transferred through plasmids in *Enterobacteriaceae* first described in 1988 [202]. FosA-class are metalloenzymes that catalyze the nucleophilic addition of the tripeptide glutathione to the fosfomycin C1 position, cleaving the epoxide ring and inactivating the antibiotic [207–209]. New subtypes, with similar structure, of the gene have been described [154]. FosA3 is the most prevalent enzyme isolated in *E. coli* clinical isolates, with an aminoacid homology of 80% with FosA [210]. FosA4 is a newly identified variant of FosA3, sharing 94% of aminoacid identity that has been recently described in *E. coli* [211]. The *fosA5* gene encodes a 139-amino-acid protein that shares 70 and 73% identity with FosA and FosA3, respectively [212(p5)]. Another FosA subtype, named fosKP96, has also been described in two *E. coli* strains from blood and urine infections, although there is little information about its activity and identity rates with other FosA enzymes [213]. Co-occurrence of FosA and FosA3 subtypes in plasmids with severeal antibiotic resistance genes have been reported, conferring resistance to β-lactams, quinolones, aminoglycosides, macrolides, sulfonamides, and tetracyclines [186,214–216]. The genes conferring resistance to fosfomycin could be transferred together with genes conferring resistance to other antibiotics in either the same or a conjugate plasmid [154].

The presence of FosC2 in *E. coli* have been recently described in Japan [210]. The amino acid sequence of FosC2 had 72% identity to that of FosC found in *Achromobacter xylosoxidans.* However, the prevalence of FosC2 in *E. coli* clinical strains remains unclear.

3.4.3.3. Low-level fosfomycin resistance:

The steps by which fosfomycin-resistant *E. coli* mutants arise and spread during UTI is not well known. As previously mentioned, fosfomycin resistance has been associated with chromosomal mutations in genes related to the fosfomycin target (*murA*) or to fosfomycin intake (*glpT*, *uhpA*, *uhpT*, *cyaA* or *ptsI*). However, there are very few studies that provide information about the prevalence of these mutations in UPEC, as well as the contribution of each mutation to fosfomycin resistance.

Ballestero-Téllez and colleagues [217] have recently published that the presence of single chromosomal mutations, or even in selected combinations, are related with increases in fosfomycin MICs, but not conferring clinical resistance according to international guidelines. However, although the presence of low-level fosfomycin resistance (LLFR) mutants yields a fosfomycin-susceptible phenotype, they may facilitate the selection of highly resistant subpopulations when additional mutations appear. There are only two studies where LLFR in *E. coli* clinical strains have been evaluated [183,192]. Nilsson and colleagues studied 13 clinical strains, seven of them showing reduced fosfomycin susceptibility (MIC ≥8μg/mL), but still considered as susceptible to fosfomycin (MIC ≤64). Ohkoshi and colleagues studied 211 *E. coli* clinical strains, where seven were LLFR. These strains showed several mutations in one or more genes (*uhpT*, *glpT*, *cyaA* and *ptsI*) leading to amino acid deletions or changes of amino acid residues, compared with other susceptible strains. However, it is unclear whether these mutations contributed to the reduced susceptibility.

3.4.3.4. Effect of urinary tract physiological conditions on fosfomycin activity

Conversely, the effect of urinary tract physiological conditions on fosfomycin activity has not been studied as well as ciprofloxacin. Hence, there is no previous evidence concerning the effect of urine compounds on fosfomycin activity. Regarding the effect of pH, the activity of fosfomycin increases at acidic pH values [142,218,219], with the greatest activity observed at pH 5.5 [220,221]. However, the molecular basis of this effect is obscure. It has been shown that acidic environments decrease cAMP levels into bacterial cytosol [222], and consequently, the expression of the fosfomycin transporters GlpT and UhpT are reduced. For this reason, fosfomycin activity should be reduced under acidic pH values, but the reality appears to be far different. Recently, Fedrigo and colleagues [223] evaluated the effect of fosfomycin at pH6 and 7 against *E. coli* and *Klebsiella pneumoniae* strains. They suggest that at acidic pH values, fosfomycin molecules are partially protonated, in a more lipophilic state, that allows the entrance of fosfomycin molecules into bacteria, thus resulting in a greater activity.

The activity of fosfomycin increases under anaerobic conditions [224]. Unlike urine and pH, molecular mechanisms related with fosfomycin activity under anaerobic conditions are well understood. The FNR is active under anaerobic conditions, binding to the regions upstream of *glpT* and *uhpT*, acting as an activator of these genes [178]. Furthermore, a higher expression of *crp* and *cyaA* genes has been found in anaerobic conditions, increasing, in turn, the expression of *glpT* and *uhpT* [225]. Wherefore, the increased antibacterial activity of fosfomycin against *E. coli* strains under anaerobic condition is attributed to an elevated expression of GlpT and UhpT transporters.

In the particular case of fosfomycin, it appears that urinary tract physiological conditions (low pH values and anaerobiosis) could increase its activity, allowing a good effect against LLFR mutants. Nevertheless, it is necessary to know how LLFR mutants contribute to fosfomycin-susceptibility and bacterial fitness under UTI conditions.

Chapter IV

Objectives

The main objective of this Thesis was to evaluate the impact of urinary tract physiological conditions (urine, pH and anaerobiosis) on the antimicrobial activity of ciprofloxacin and fosfomycin against *E. coli*. The research is divided into a series of partial objectives that can be summarized as follows:

1. To study the effect of urinary tract physiological conditions on ciprofloxacin activity against a set of well-characterized isogenic LLQR *E. coli* strains, carrying the most frequent chromosomal mutations and PMQR determinants.

1.1 To evaluate the effect of LLQR mutations on bacterial growth rates under urine conditions.

1.2. To determine the effect of urine, pH and anaerobiosis on ciprofloxacin MIC values on well-characterized LLQR isogenic strains.

1.3. To evaluate the survival rates of these strains in the presence of the maximum concentration of ciprofloxacin reached in the bladder.

1.4. To study the effect of urine, pH and anaerobiosis on ciprofloxacin activity against well-characterized LLQR clinical isolates.

2. To study the effect of urinary tract physiological conditions on fosfomycin activity against a set of well-characterized isogenic LLFR and fosfomycin-resistant strains, carrying the most frequent chromosomal mutations.

2.1. To evaluate the effect of LLFR mutations on bacterial growth rates under urine conditions.

2.2. To determine the effect of urine, pH and anaerobiosis on fosfomycin MIC values on well-characterized LLFR isogenic strains.

2.3. To study the effect of urine, pH and anaerobiosis on fosfomycin activity against well-characterized LLFR and fosfomycin-resistant clinical isolates.

Chapter V

Publications

'Results! Why, man, I have gotten a lot of results!
I know several thousand things that won't work.'

-Sir Thomas A. Edison.

Publication I: Urinary Tract Physiological Conditions Promote Ciprofloxacin Resistance In Low-Level Quinolone Resistant *Escherichia coli.*

An increase in resistance to fluoroquinolones has been described worldwide during recent years. DNA gyrase and DNA topoisomerase IV, involved in DNA supercoiling, are the targets of fluoroquinolones. Gyrase and topoisomerase IV are composed of two subunits encoded by the genes *gyrA* and *gyrB*, and *parC* and *parE*, respectively. Ciprofloxacin resistance can be acquired through mutations in these genes encoding gyrase and topoisomerase or in genes affecting efflux or permeability of the bacteria such as *marR* or *acrR*. Typically, single mutations have been associated with low-level fluoroquinolone resistance, and accumulation of several mutations develops high-level resistance. Some of these mutations confer LLQR with slightly increased MICs than is common for the susceptible population, but still under the resistant breakpoint. Remarkably, LLQR strains have been suggested to be the first evolutionary step for producing high-level of quinolone resistance.

The main objective of this work was to elucidate the effect of urinary physiological conditions on ciprofloxacin activity against *E. coli* strains carrying LLQR mutations. Ciprofloxacin is an ampholyte that can exist in four different pH-dependent protonation forms. Thus, depending on the pH, the ionization status of ciprofloxacin increases its permeability or improves its intestinal absorption modifying consequently its antimicrobial activity. Furthermore the presence of urinary divalent cations and anaerobic conditions have been related with a reduction of ciprofloxacin activity. The combination of ciprofloxacin activity reduction in the urinary tract and the presence of LLQR mutants may lead to a non-lethal selection, where the bacterial growth is inhibited but they are not killed.

Our results demonstrate that urinary tract physiological conditions reduce significantly the effect of ciprofloxacin against *E. coli* strains with LLQR mutations. The presence of urine, low-pH values and anaerobiosis increase the MIC values of ciprofloxacin, with all the LLQR mutants becoming clnically resistant strains according to international guidelines. Furthermore, the presence of these mutations also facilitates high bacterial survival even when we simulate the maximum ciprofloxacin concentration reached in urine within the first 6 h after administration of an oral dose of 500 mg of ciprofloxacin.

The results presented here raise questions concerning the general applicability of the recommended methods for MIC determinations, which appears to be a poor estimator of ciprofloxacin activity against LLQR *E. coli* in UTIs. International guidelines for MIC determinations should reconsider clinical breakpoints, taking into account the effect of common low pH values in ciprofloxacin activity.

Guillermo Martín-Gutiérrez; Jerónimo Rodríguez-Beltrán; José Manuel Rodríguez-Martínez; Coloma Costas; Javier Aznar; Álvaro Pascual; Jesús Blázquez. Urinary tract physiological conditions promote ciprofloxacin resistance in low-level quinolone resistant *Escherichia coli*. ***Antimicrobial Agents and Chemotherapy*** 2016, 20: 60(7):4252-8.

Publication II: Plasmidic *qnr* Genes Confer Clinical Resistance to Ciprofloxacin under Urinary Tract Physiological Conditions.

Taking the previous proposal (**Publication I**) as a premise, the aim of this work was to evaluate the impact of urinary physiological conditions on ciprofloxacin activity against *E. coli* strains harboring plasmidic *qnr* genes. Plasmid genes *qnrA*, *qnrB*, *qnrC*, *qnrD*, and *qnrS* code for proteins of the pentapeptide repeat family that protect DNA gyrase and topoisomerase IV from quinolone inhibition. The presence of PMQR genes lead to generation of LLQR, with fluoroquinolone MICs that are higher than the epidemiological cutoff value but still below the resistance breakpoint for most fluoroquinolones.

The *qnr* genes have apparently to have been acquired from chromosomal genes in aquatic bacteria, and are usually associated with mobilizing or transposable elements on plasmids. Additionally, *qnr* genes are often associated with extended-spectrum β-lactamases and aminoglycoside-resistance genes on the same plasmid, and thus, increase the probability of survival after UTI treatment and dissemination.

The results presented here show how the presence of urine, low-pH values and anaerobiosis lead to reduce ciprofloxacin activity, causing an increase of MIC values above the cutoff of resistance. Thus, UTI conditions could be an ideal environment for the selection of strains harboring *qnr* genes, allowing the survival of *E. coli* strains considered susceptible to fluoroquinolones. Again, international MIC breakpoints appear to be poor estimators of ciprofloxacin activity against LLQR strains.

Guillermo Martín-Gutiérrez; José Manuel Rodríguez-Martínez; Álvaro Pascual; Jerónimo Rodríguez-Beltrán; Jesús Blázquez. Plasmidic *qnr* Genes Confer Clinical Resistance to Ciprofloxacin under Urinary Tract Physiological Conditions. ***Antimicrobial Agents and Chemotherapy*** 2017, 24: 61(4): pii: e02615-16.

Publication III: Urinary tract conditions affect fosfomycin activity against *Escherichia coli* strains harboring chromosomal mutations involved in fosfomycin uptake.

Although fosfomycin is one of the most commonly used treatments for UTIs, the steps by which *E. coli* strains harbouring mutations related with fosfomycin-resistance arise and spread is not well understood. Fosfomycin resistance can be achieved by reducing permeability to fosfomycin through mutations in genes encoding the GlpT and UhpT transporters. Fosfomycin permeability can also be reduced by mutations in *cyaA* and/or *ptsI* genes, which regulate the intracellular cAMP levels necessary for fosfomycin-transporter activation. The presence of single chromosomal mutations and some of their combinations confer LLFR, but not clinical resistance, acting as a gateway for highly resistant subpopulations by the selection of additional LLFR mutations.

However, there is a very low prevalence of fosfomycin-resistant *E. coli* strains from UTIs. This low prevalence may suggest that LLFR mutants will also present a very low prevalence. Fosfomycin-resistance is associated with a high biological cost, with decreased growth rates as well as decreased virulence.

The objective of this work was to evaluate the impact of urinary tract physiological conditions on the antimicrobial activity of fosfomycin against a set of well-characterized isogenic strains harbouring deletions in the genes most frequently related with fosfomycin-resistance (Δ*glpT*, Δ*uhpT*, Δ*cyaA*, Δ*ptsI*). Additionally we evaluated the impact of UTI conditions on bacterial fitness. A series of fosfomycin-resistant *E. coli* clinical strains isolated from patients with UTI were also studied.

The results presented here demonstrate that urinary tract conditions might have a profound impact on fosfomycin activity. The presence of low-pH values in urine and

anaerobic conditions increased fosfomycin activity, allowing the eradication of strains harbouring fosfomycin-resistance mutations. Furthermore, the presence of single and double-deletions were related with a significative decrease in maximal growth rates under urinary conditions. This work adds a new explanation to the low prevalence of *E. coli* fosfomycin-resistant variants in UTIs.

Guillermo Martín-Gutiérrez, Fernando Docobo-Pérez, Jerónimo Rodriguez-Beltrán, José Manuel Rodríguez-Martínez, Javier Aznar, Álvaro Pascual, Jesús Blázquez. Urinary tract conditions affect fosfomycin activity *against Escherichia coli* strains harboring chromosomal mutations involved in fosfomycin uptake. ***Antimicrobial Agents and Chemotherapy*** 2017, 21: 62(1): pii: e01899-17

Chapter VI

General discussion

Multiple resistance to antibiotics represents a global health challenge that results in increased morbidity and mortality rates all over the world [32]. For instance, the increasing prevalence of antibiotic-resistant uropathogens is likely to limit the effectiveness of our current therapeutic choices. Thus, UTIs are becoming increasingly difficult to treat owing to the widespread emergence of antibiotic resistance mechanisms [226]. Furthermore, the high rates of recurrence in UTIs caused by susceptible uropathogens suggest that some antibiotics are not as effective as we thought. In this context, the current emergence of antibiotic-resistant strains, in conjunction with the high frequency of recurrent UTIs, emphasize the need for a better understanding of UTIs, as well as the effectiveness of antibiotics under physiological UTI conditions. On this basis, the main objective of the present thesis has been to evaluate the effect of urinary tract physiological conditions on two of the most used antibiotics for the treatment of UTIs caused by *E. coli*: ciprofloxacin and fosfomycin.

The research presented in this thesis set out to identify the critical factors existing in the urinary tract that might promote or facilitate the survival of LLR mutants during therapeutic treatments. The main relevance of LLR strains is that they are considered an intermediary stage towards the development of high-resistance, carrying mutations or resistance genes that may result in a decrease in the effectiveness of the antibiotic [39]. Unfortunately, there is not a standard definition for LLR in international clinical guidelines. MIC is defined as the minimal concentration of drug that prevents visible bacterial growth under strictly defined *in vitro* conditions, being measured using increasing concentration steps, with either broth dilution or gradient MIC assays [227]. Thus, development of resistance can be considered the consequence of any genetic changes that increase the MIC of a strain to a level higher than the wild-type MIC/ecological cut-off. In contrast, clinical resistance is well defined and refers to the

consequences of genetic changes that increase the MIC of a strain to a level high enough to prevent successful treatment with standard drug therapy [228]. In this way, LLR mutants are traditionally categorized as susceptible strains (MIC lower than the clinical breakpoint, and standard therapy likely to be successful), and hence are is difficult to detect by most standard susceptibility testing in Clinical Microbiology Laboratories [39]. Furthermore, LLR strains appear to be a gate for the acquisition of high-level clinical resistance, which may have important consequences for the treatment and control of UTIs caused by these microorganisms.

MIC determination gives a measure of growth inhibition under specific *in vitro* conditions, and its clinical usefulness requires that these MIC values would be able, in some way, to be translated into a prediction of the clinical outcome [229]. Mueller-Hinton (MH) broth is the medium of choice for susceptibility testing of commonly isolated aerobic or facultative organisms [230]. This medium shows acceptable batch-to-batch reproducibility and low concentration of inhibitors, with a stable pH value from 7.2 to 7.4, supporting satisfactory growth of most common pathogens [231]. Nonetheless, the situation in which antibiotics act under physiological conditions is quite different.

Under physiological conditions, antibiotics interact with a set of proteins, cations, changing pH values and variable oxygen concentrations. As was mentioned in the Introduction (**Chapter III**), these conditions may compromise the activity of many antimicrobials, allowing the survival of a significant number of microorganisms. Despite this fact, little attention has been paid to the role of the physiological conditions during treatment. In the urinary tract, antibiotics face the presence of urine compounds, changing pH values and an oxygen-reduced concentration. To date, most of studies have focused on evaluating the effect of pH and urine on quinolone effectiveness. For

instance, Zeiler HJ [232] studied the influence of pH and human urine in quinolone susceptibility testing, showing that acidic pH was related to lower quinolone activity. Zhanel and colleagues [233] have also showed the reduction of ciprofloxacin activity under low-pH values in urine. Recently, other studies have presented similar results [142]. However, there are few studies evaluating the effect of physiological urinary conditions in other antibiotics like fosfomycin. Furthermore, the effect of anaerobic conditions in combination with urine and low-pH has been overlooked in these studies.

Of special interest is the impact of the urinary tract conditions and the presence LLR mutants during UTI treatment. The presence of LLR strains in the bladder could increase the possibility of the emergence of a clinically resistant phenotype to relevant antibiotics. Regrettably, few studies have shown the therapeutic impact of LLR in UTIs, either experimentally or in clinical reports.

In **Publications I** and **II** we sought to evaluate the impact of the urinary tract physiological conditions (namely, growth in urine, pH, and anaerobiosis) on the antimicrobial activity of ciprofloxacin against a set of well-characterized isogenic LLQR strains carrying the most frequent chromosomal mutations and PMQR determinants. We have demonstrated that urinary tract physiological conditions could be an optimum scenario for the selection of strains harboring CMQR and PMQR genes, decreasing their susceptibility to ciprofloxacin and allowing the survival of *E. coli* strains that have traditionally been considered susceptible. This effect was also observed in LLQR mutants isolated from patients with UTI. It has been previously thought that multiple mutations are required to generate clinical ciprofloxacin resistance in *E. coli* strains. Nevertheless, our work shows how urinary tract conditions may provide an ideal scenario for the generation and selection of LLQR with single mutations or *qnr* genes,

which would allow its survival during ciprofloxacin treatmen under physiological concentrations, even though LLR strains were considered susceptible to fluoroquinolones. Furthermore, it has been demonstrated that a strong positive correlation between increased mutation rate and successful accumulation of quinolone resistance–associated mutations [98] exists in *E. coli* strains isolated from patients with UTI. In this manner, the presence of chromosomal mutations like GyrA S83L or plasmidic Qnr determinants increase the mutant prevention concentration, facilitating the selection of additional resistance mutations [234].

It should also be emphasized that fluoroquinolone antibiotics stimulate two important bacterial pathways to produce genetic variation: mutagenesis and recombination [149,235.] Ciprofloxacin produces double-strand breaks, potentially lethal DNA lesions that occur under physiological conditions through collapse of stalled replication forks, overlapping repair tracts or spontaneous breakage of DNA [236]. *E. coli* efficiently repairs double-strand breaks through a series of reactions carried out by enzymes participating in homologous recombination and replication that are induced by the SOS system. Thereby, ciprofloxacin acts as an inducer of the SOS system, which leads to an increase in mutagenesis, recombination and/or horizontal gene transfer, key processes for the emergence and spread of resistance [237]. Therefore, fluoroquinolones can be viewed as a double-edged sword that may promote the generation of antibiotic resistance.

It is also important to notice that *E. coli* strains growing in the urinary tract are frequently under stress because they are starved, under antibiotic treatment, or challenged by the need to colonize novel environments with low pH values or under the inhibitory effects of host defense mechanisms [238]. Justice and colleagues demonstrated

that the ability to form filaments in bladder cells, an important virulence property that facilitates persistence in the murine cystitis model [239], is the result of bacterial SOS response induction. This result demonstrates that the SOS system is activated during bladder cell invasion. Thus, mutation and recombiation rates are probably much higher in the course of a UTI process than those determined *in vitro*. Furthermore, the urinary tract represents a heterogeneous environment, where bacteria are frequently attached to cell surfaces or inside the host cells. The presence of different microenvironments allows the emergence and co-existance of different phenotypes, which may promote higher degrees of antibiotic-resistant mutations.

Taken together, the arguments presented above let us to propose that prevalent mutations conferring LLQR phenotypes could provide the basal level of resistance needed to withstand the first hours during ciprofloxacin treatment under UTI conditions. Then, the surviving population will benefits from increased mutation and recombination rates, providing a window for the emergence of additional resistance. Therefore, ciprofloxacin might trigger a phenomenon known as "directional selection" (**Figure 10**), in which the susceptible phenotypes would be at a disadvantage compared to the more resistant phenotypes [240]. This will cause a shift in the population towards more resistant strain**s**, increasing their proportion. In our case, this effect is enhanced by the presence of LLQR strains and urinary physiological conditions, which reduce ciprofloxacin activity. This effect could explain, at least in part, the high rates of fluoroquinolones resistance in *E. coli* isolates from patients with UTI. In this way, it has been reported that patients previously treated with fluoroquinolones are more prone to urinary tract infection caused by ciprofloxacin-resistant *E. coli* strains [241]. The study performed by Ena and colleagues [241] showed that previous treatment with quinolones, urinary abnormalities, patient age higher than 64 years and the presence of a urinary

catheter were independent factors related to infecions caused by quinolone-resistant microorganisms. Similar results have been reported in more recent studies [86,88,242].

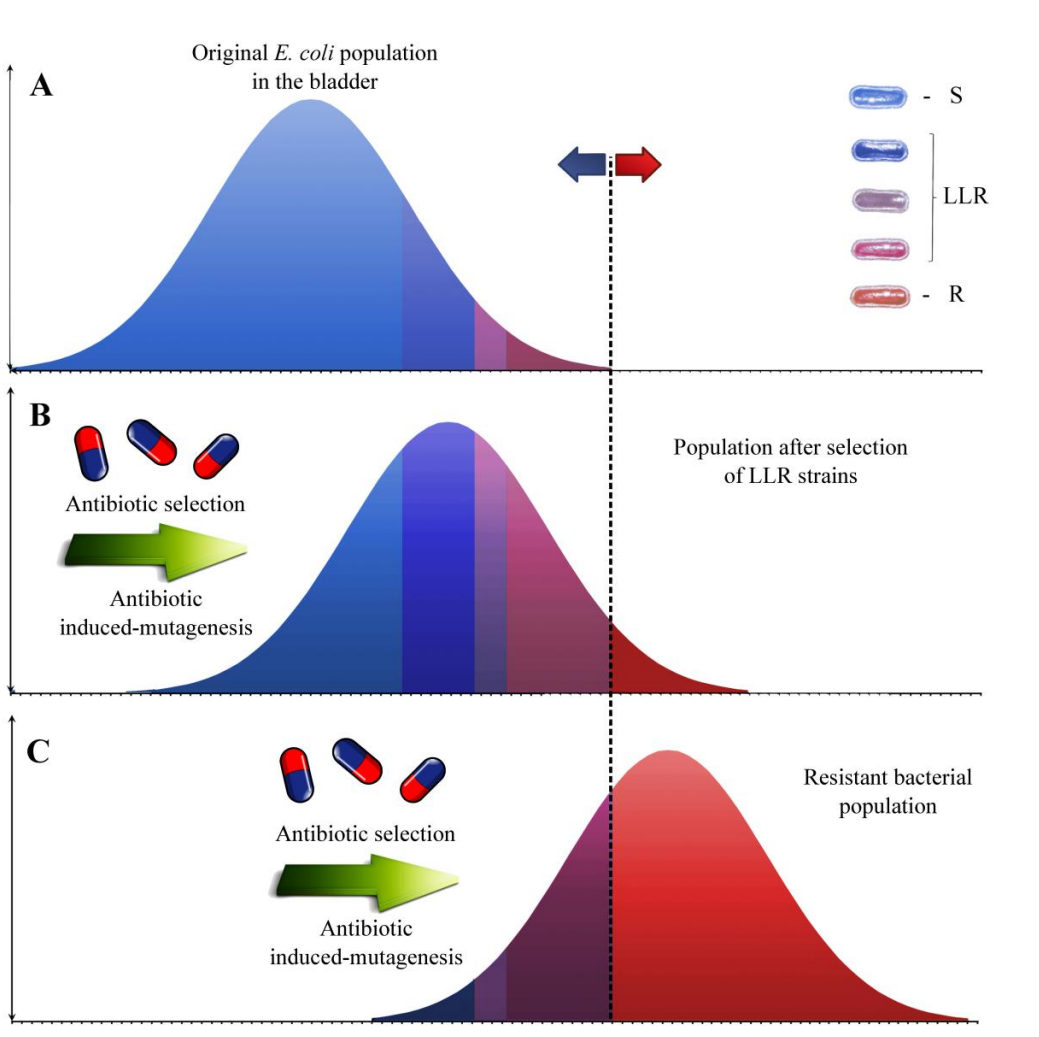

Figure 10. Directional selection as a product of antibiotic treatment under urinary tract physiological conditions. **A**) Initial population of susceptible *E. coli* strains in the urinary tract, with the presence of LLR mutants. **B & C**) If antibiotic activity is reduced under UTI conditions, LLR strains could be selected. In this sense, the antibiotic contributes to a directional selection, shifting the original susceptible population. Moreover the mutagenesis effect of antibiotics might contribute to the development of high-resistant strains.

Andersson and Hughes [33] suggested that most resistance mechanisms incur a fitness cost for the bacterium, which is manifested by a decreased bacterial growth rate. Antibiotics target important functions such as cell wall synthesis or regulation of

chromosome supercoiling, hence mutations in genes participating in these processes arguably cause a reduction in fitness [243]. To evaluate the impact of CMQR mutations on bacterial fitness, we studied the growth rates of these strains. Previously, Machuca and colleagues [244] showed that no significant differences, in terms of maximal growth rate, were observed during the exponential phase in a set of isogenic strains carrying the most frequent chromosomal mutations related with quinolones resistance. This experiment was performed in Lysogeny Broth (LB) and M9 media. Additionally, fitness cost was also measured with competition experiments using a murine model of systemic infection. Commonly, fitness experiments are performed in rich or in defined minimal media, which differs with real physiological conditions [245.] Fitness is relative and should be measured in physiological environments in order to establish its predictive value in determining the clinical outcome of infection [243]. Therefore, a correct estimation of the potential fitness cost of antimicrobial resistance is an essential factor for predicting the risk of resistance development in the urinary tract [90]. For this reason, we performed growth curves in MH broth and urine at pH values of 7, 6 and 5, using an automated spectrophotometer under controlled conditions of temperature and shaking. The patterns of the curves obtained (**Annexe II**) were similar for all isogenic derivates within the same medium, showing that the LLQR mutations studied have a reduced effect in fitness, being the strains able to grow in urine at different pH values. Acidification of urine, including pH 5, led to only small decreases in growth, demonstrating that *E. coli* is well adapted to extreme pH values.

The third part of this thesis aimed to investigate the effect of urinary tract physiological conditions on fosfomycin activity. The steps by which *E. coli* strains harboring mutations related with fosfomycin resistance arise and spread during UTI is not well known. For this reason, in **Publication III** we evaluated the effect of urine, pH

variation and anaerobiosis on fosfomycin MIC values against a set of isogenic strains carrying the most prevalent chromosomal mutations conferring fosfomycin-resistance and their combinations.

The first concern that we found during this work was the lack of information about the prevalence of LLFR mutations in *E. coli* isolates from UTI, as well as the identification of the molecular mechanisms related with fosfomycin-resistance patterns. Nilsson and colleagues [183] performed the first study where fosfomycin-resisatance chromosomal mutations related were studied, analysing thirteen *E. coli* fosfomycin-resistant strains from UTIs. In four isolates, gene-inactivating mutations were found in the *uhpT* and/or *uhpA* gene; and one strain carried a mutation in *glpT* as well as a deletion in *uhpA*. However, in eight strains the mechanisms of fosfomycin resistance were not identified. Similar results were obtained by Takahata and colleagues [246], where the lack of an entire *uhpT* gene and the insertion of an ISEcp1 element in the *glpT* gene were the most prevalent mutations in six *E. coli* fosfomycin-resistant clinical strains. In two further publications, the presence of fosfomycin-resistance mutations were studied in ESBL-producing *E. coli* strains, showing a wide range of mutations in *uhpT*, *glpT*, *uhpA*, and *murA* genes, as well as the presence of FosA [193,216,247]. There are only two publications where LLFR strains have been studied, showing several mutations and deletions in *uhpT*, *glpT*, *cyaA* and *ptsI* genes [183,192].

Although mutations in *uhpT*, *glpT*, *cyaA* and *ptsI* genes seem to be the main sources of fosfomycin resistance, there is no identified mutation-pattern of fosfomycin resistance. Unlike ciprofloxacin, the mutations and deletions found in fosfomycin-resistant strains from previous works are largely different. Due to the lack of correlation, the impact of each mutation or partial deletion should be carefully studied.

In **Publication III**, we included the strain *E. coli* BW25113 and ten isogenic strains carrying the most prevalent single (Δ*uhpT*, Δ*glpT*, Δ*cyaA* and Δ*ptsI*) and double deletions (Δ*glpT*-Δ*uhpT*, Δ*glpT*-Δ*cyaA*, Δ*glpT*-Δ*ptsI*, Δ*uhpT*-Δ*cyaA*, Δ*uhpT*-Δ*ptsI* and Δ*ptsI*-Δ*cyaA*) in fosfomycin-resistance genes [217]. In order to exclude the possibility that the effects observed were specific to the strain BW25113, we evaluate the effect of urinary tract physiological conditions in five *E. coli* clinical isolates from patients with UTI. Overall, our results demonstrate that urinary tract physiological conditions might have a profound impact on fosfomycin activity against strains with fosfomycin-resistance mutations. Specifically, acid pH values and anaerobiosis have a great effect on fosfomycin activity, converting most of the strains categorized as resistant, according to the international guidelines, into susceptible. Nonetheless, the presence of urine increases the MIC values for some strains, although this effect is attenuated under acidic conditions and incubation under anaerobic conditions. We showed the effect of single and double-deletions on genes related with fosfomycin-resistance under physiological conditions, confirming that standard susceptibility testing could underestimate the effect of fosfomycin, mainly in patients with acid pH values in urine.

Fosfomycin resistance has been previously related with a high biological cost, entailing a generally reduced fitness that compromises competition with the normal microbiota in the human host [182]. The effect of fosfomycin-resistance mutations on fitness is of particular interest in UPECs, because if the cost is high enough, the resistant bacteria will not growth on the minimal rate needed to stablish an infection in the bladder [183,248]. As was previously mentioned, to obtain a correct estimation of the potential fitness cost, we determined the maximal growth rates per hour in urine at different pH values for all isogenic strains, comparing the results obtained with those in MH. As can be observed in **Publication III**, all strains with single and double deletions

showed statistically significant decreases in growth rates when grew in urine at different pH values, compared with the strain BW25113. This result is in agreement with previous studies which demonstrated that fosfomycin-resistant strains show lower growth rates [183], decreased adhesion to uroepithelial cells as well as urinary catheters [182], and alteration in cell surface hydrophobicity [249]. The possible causes of these defects in bacterial physiology are likely attributed to the pleiotropic effects caused by fosfomycin resistance mutations. For instance, mutations in *cyaA* and *ptsI* genes lead to lowered cAMP levels, which imply lower expression of *uhpT* and *glpT*, and thus, increased fosfomycin resistance. However, cAMP is a key metabolite in bacterial physiology and the perturbation of its level is expected to drastically alter bacterial homoestasis [250]. In this line, it has been stated that a reduction of cAMP levels cause a reduction in pilus biosynthesis, reducing the virulence factors in fosfomycin-resistant strains [183]. It should be noted that fitness cost of fosfomycin resistance needs to be measured for each particular species, being the observations made in just one species not extrapolable to another [251]. The uptake of fosfomycin in *Pseudomonas aeruginosa* depends exclusively on GlpT transporter, which does not seem to be regulated by cAMP levels. Thus, fosfomycin resistance in *P. aeruginosa* was related with an absence of *in vivo* fitness cost [251].

In the other hand, only increased MurA levels have been correlated with higher levels of fosfomycin resistance at a low fitness cost in *E. coli* [200]. However, to date no clinical studies reporting strains with high MurA levels have been published. Moreover, as mentioned in **Chapter III**, there are few reports of clinical isolates showing mutations in the *murA* gene, and none in the catalytic site of MurA, because most of them reduce drastically bacterial cell viability.

In contrast with ciprofloxacin, treatment with fosfomycin does not activate the SOS system in *E. coli* [200]. Thus, it has been demonstrated that fosfomycin treatment does not increase the number of fosfomycin-resistant mutants [252] nor the homologous recombination rates [253]. Furthermore, the low prevalence of fosfomycin-resistant *E. coli* strains from patients with UTI may suggest that the prevalence of LLFR strains should be very low. If we combine the previous considerations with the high-fitness cost of these mutations and the increment of fosfomycin activity under UTI conditions, it leads to exactly the opposite situation of what was described in Figure 10. In this way, our results demonstrate that urinary tract physiological conditions might have a profound impact on fosfomycin activity against strains with fosfomycin-resistance mutations, adding a new explanation to the low prevalence of *E. coli* fosfomycin-resistant variants in UTIs.

Concerning the clinical and physiological characteristics of patients with UTI caused by *E. coli*, there are several factors associated with urine pH modification that should be taken into consideration when the treatment is chosen. Pregnant women have an increased glomerular filtration rate and higher urinary calcium excretion throughout pregnancy, with higher urine pH values in the second and third trimesters [254]. Thiazide diuretic intake is also associated with a higher urine pH by reducing the urinary uric acid excretion [255]. Furthermore, there are genetic disorders that are related with urine alkalization. Thus, Gitelman syndrome is an autosomal recessive disorder of the thiazide-sensitive sodium chloride cotransporter, expressed at the distal convoluted tubule, which is accompanied by an inappropriately high urine pH [256]. In these cases, if fosfomycin is the treatment of choice, the presence of LLFR could lead to treatment failure and the development of fosfomycin resistance. Thus, urinary pH values could have practical interest in the management of these patients, where the physician should

select the course best suited to the individual patient.

On the other side, there are also clinical factors related with urine acidification. It has been reported that the composition of the diet affects acid-base balance in the body. A higher protein intake significantly increases the renal acid excretion by increasing ammonium output, thus acidifying the urine [257, 258]. Moreover, the effect of urine pH on ciprofloxacin and fosfomycin activity may become relevant in patients with certain underlying characteristics or diseases. It is known that patients with hypertension are associated with a lower urinary citrate and higher acid excretion, resulting in lower urine pH values [259]. This effect can also be found in patients with type 2 diabetes or metabolic acidosis [260]. There are some patient characteristics that are related with lower pH values in urine, like older age or higher body weight [261,262]. Some medications can also acidify urine, such as loop diuretics or mineralocorticoids [263,264]. Consequently, if ciprofloxacin is the treatment chosen in patients with factors related to urine acidification, and the UTI is produced by LLQR mutants, it could result in therapeutic failure and increased selective pressure, promoting the development of bacterial resistance.

Another posibility is modifying the urine pH of patients with UTI, either increasing urine pH by alkalinisation (e.g. by the use of potassium citrate or sodium bicarbonate [265,266]) with the aim of increasing the activity of ciprofloxacin, or by acidifying the urine, with the aim of increasing the activity of fosfomycin. However, urinary pH modification is frequently difficult to achieve and is rarely, if ever, necessary. To acidify the urine, it is often necessary to modify the diet by restriction of agents that tend to alkalinize the urine (milk, fruit, juices, sodium bicarbonate) [1]. Another major problem with acidification is that patients with renal insufficiency are

unable to excrete an acid load, and may become systematically acidotic when urinary acidification is attempted [267]. Similar problems could be found with urine alkalinization [268]. For this reason, the best choice should be the selection of the antibiotic therapy according to the clinical characteristics of the patient.

Chapter VII

Conclusions

The studies developed in this Thesis have led to results that allow the following main conclusions:

1. Urinary tract physiological conditions have a profound impact on ciprofloxacin and fosfomycin activity against *E. coli* strains harboring the most prevalent LLR mutations.

2. The presence of urine, low-pH and anaerobiosis reduce ciprofloxacin activity. These conditions generate an ideal environment for the selection of strains harboring LLQR determinants, decreasing their susceptibility to ciprofloxacin, allowing for the survival of microorganisms traditionally considered as susceptible.

3. Despite the general belief that multiple mutations are required to generate clinically important resistance in *E. coli,* the results presented here show that LLQR mutants are capable for surviving under physiological concentrations of ciprofloxacin in the bladder.

4. The presence of acid pH values and anaerobiosis have a great effect on fosfomycin activity, converting most of the strains categorized as resistant, according to the international guidelines, into susceptible.

5. The effects of urine, pH and anaerobiosis observed in the isogenic strains have been corroborated in clinical strains with LLR and high-resistance mutations.

6. Internationally recommended methods for MIC determination produce poor estimations of ciprofloxacin and fosfomycin activity against LLR *E. coli* strains under urinary physiological conditions. Under this assumption, international guidelines should prompt a reconsideration of clinical breakpoints, taking into account physiological conditions during infection.

7. Urinary pH values could have practical interest in the management of patients with UTI, where the physician should select the course best appropriate to the individual patient.

Chapter VIII

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Chapter IX

Annexes

Annexe I: Additional scientific production

Congress participation:

- ∅ Las condiciones fisiológicas del tracto urinario afectan la actividad de fosfomicina frente a cepas de *Escherichia coli* con mutaciones en genes implicados en su susceptibilidad. **Guillermo Martín-Gutiérrez**, Fernando Docobo, Jerónimo Rodríguez-Beltrán, José Manuel Rodríguez, Javier Aznar, Álvaro Pascual y Jesús Blázquez. ***XXI Congreso Nacional de la SEIMC,*** Málaga (2017).

- ∅ Urinary tract physiological conditions as a conductive environment to avoid fosfomycin resistance in *Escherichia coli*. **Guillermo Martín-Gutiérrez**, Fernando Docobo, Jerónimo Rodríguez-Beltrán, José Manuel Rodríguez, Javier Aznar, Álvaro Pascual y Jesús Blázquez. **X Jornadas Científicas de la Red Española de Investigación en Patología Infecciosa**, Sevilla (2016).

- ∅ La sensibilidad a ciprofloxacino de cepas de *Escherichia coli* portadoras de determinantes plasmídicos de bajo nivel de resistencia a quinolonas disminuye en orina a pH ácido y anaerobiosis. **Guillermo Martín-Gutiérrez**; Jerónimo Rodríguez-Beltrán; José Manuel Rodríguez-Martínez; José Antonio Lepe; Álvaro Pascual; Javier Aznar; Jesús Blázquez**.** ***XIX Congreso Nacional de la SEIMC***, Sevilla (2015).

- ∅ Resistencia a ciprofloxacino encepas de *Escherichia coli* con mutaciones cromosómicas que confieren bajo nivel de resistencia a quinolonas dependiente de orina, pH y oxígeno. **Guillermo Martín-Gutiérrez**; Jerónimo Rodríguez-Beltrán; José Manuel Rodríguez-Martínez; María del Mar Redero-Cascón; José Antonio Lepe; Álvaro Pascual; Javier Aznar; Jesús Blázquez. ***XIX Congreso Nacional de la SEIMC***, Sevilla (2015).

- ∅ Ciprofloxacin susceptible-mediated quinolone resistant *Escherichia coli* strains are ciprofloxacin resistant in urine at acidic pH. **Guillermo Martín-Gutiérrez**; Jerónimo Rodríguez-Beltrán; José Manuel-Rodríguez; Jose Antonio Lepe; Javier Aznar; Álvero Pascual; Jesús Blázquez. ***25th European Congress of Clinical Microbiology and Infectious Diseases*** in Copenhagen, Denmark (2015).

- ∅ pH and anaerobiosis promote ciprofloxacin resistance in low-level chromosomally-mediated quinolone resistant *Escherichia coli* in urine . **Guillermo Martín-Gutiérrez**; Jerónimo Rodríguez-Beltrán; José Manuel Rodríguez; Jose Antonio Lepe; Javier Aznar; Álvaro Pascual; Jesús Blázquez. ***25th European Congress of Clinical Microbiology and Infectious Diseases*** in Copenhagen, Denmark (2015).

Conferences:

- ∅ **Title**: Las condiciones del tracto urinario modulan la susceptibilidad de *Escherichia coli* a ciprofloxacino y fosfomicina.

  Scientific event: Seminario Programa de Enfermedades Infecciosas y del Sistema Inmunitario.

  Organizer: Instituto de Biomedicina de Sevilla.

  Centre: Instituto de Biomedicina de Sevilla.

  Date: 03/10/2016

- ∅ **Title:** Actividad antibiótica y respuestas adaptativas de *Escherichia coli* en orina.

  Scientific event: IX Jornada Científicas de la Red Española de Investigación en Patología Infecciosa.

  Organizer: Red Española de Investigación en Patología Infecciosa.

  Centre: University Hospital Ramón y Cajal, Madrid.

  Date: 19/11/2014

Annexe II

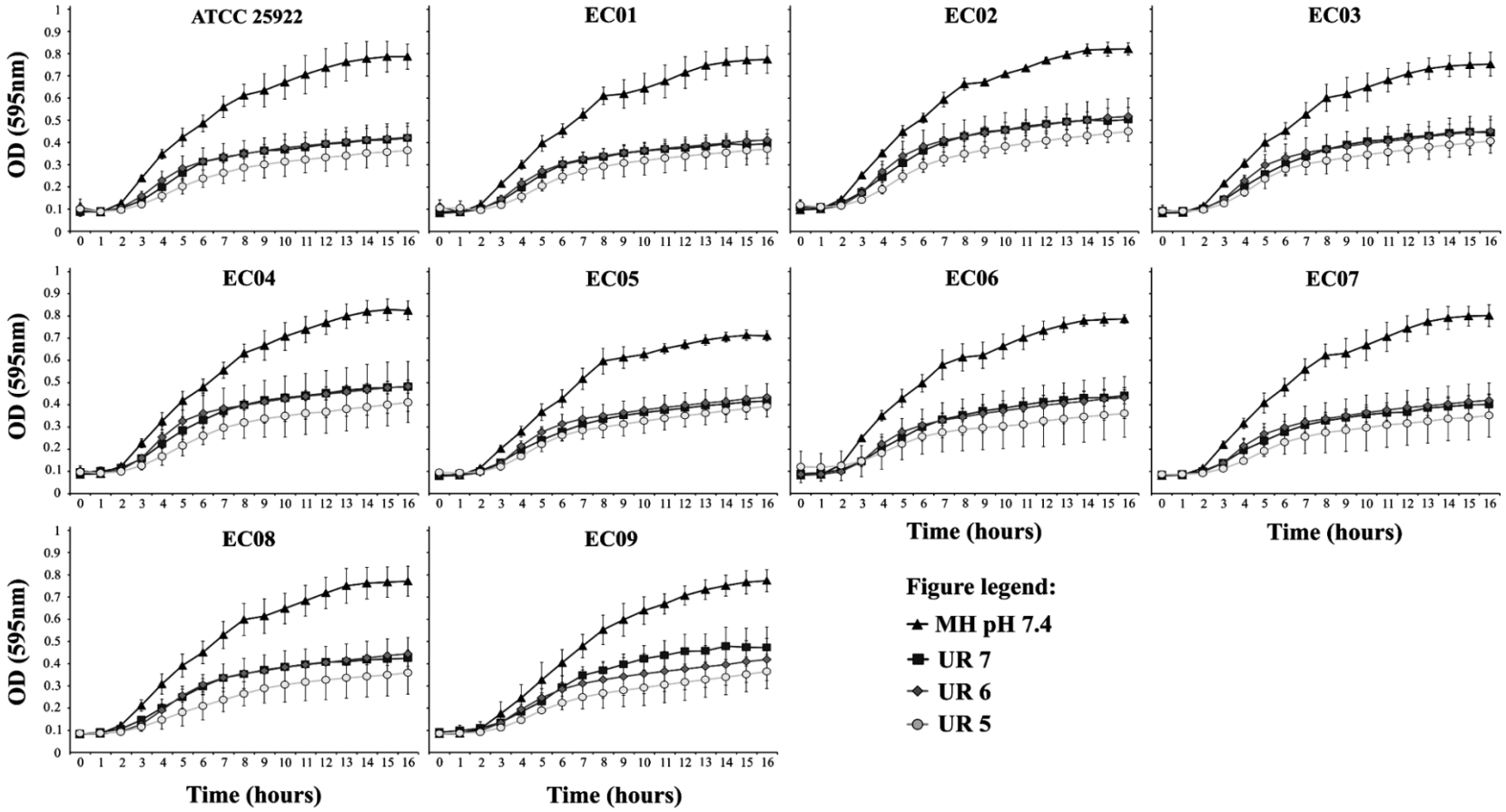

Figure 11: Growth curves of ATCC 25113 and nine isogenic strains in MH and urine at pH 7, 6 and 5. **EC01:** Δ*marR;* **EC02:** *gyrA* (S83L); **EC03**: Δ*marR-gyrA*(S83L); **EC04**: *gyrA*(S83L)–*parC*(S80R); **EC05**: Δ*marR-gyrA*(S83L)-*parC*(S80R); **EC06**: *gyrA*(S83L)-gyrA(D87N); **EC07**: Δ*marR-gyrA*(S83L)-gyrA(D87N); **EC08:** *gyrA*(S83L)-gyrA(D87N)-*parC*(S80R); **EC09**: Δ*marR-gyrA*(S83L)-gyrA(D87N)-*parC*(S80R). Error bars represent standard errors of the means of re- sults from at least four replicates. MH, Mueller-Hinton; UR, urine; OD, optical density.

Annexe III

Publication I, additional figure.

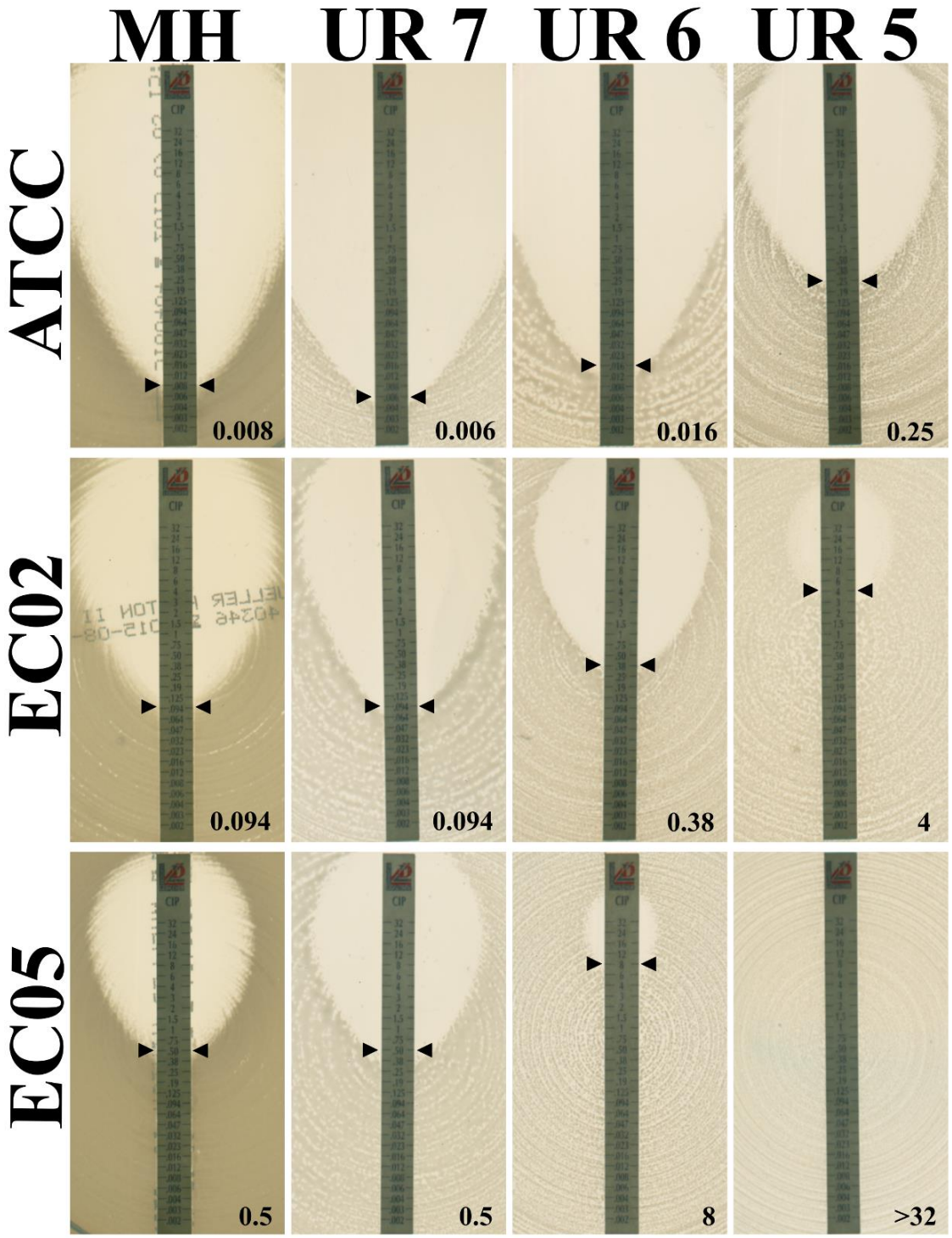

Figure 12: Ciprofloxcin susceptibility testing for the strains ATCC 25922, EC02 (*gyrA* S83L) and EC05 (Δ*marR, gyrA* S83L, *parC* S80R) using the gradient MIC strip methodology. MICs (µg/mL) are indicated at the right bottom for each condition. MH: Mueller-Hinton agar; UR7: urine-agar pH7; UR6: urine-agar pH6; UR5: urine-agar pH5.

Annexe IV

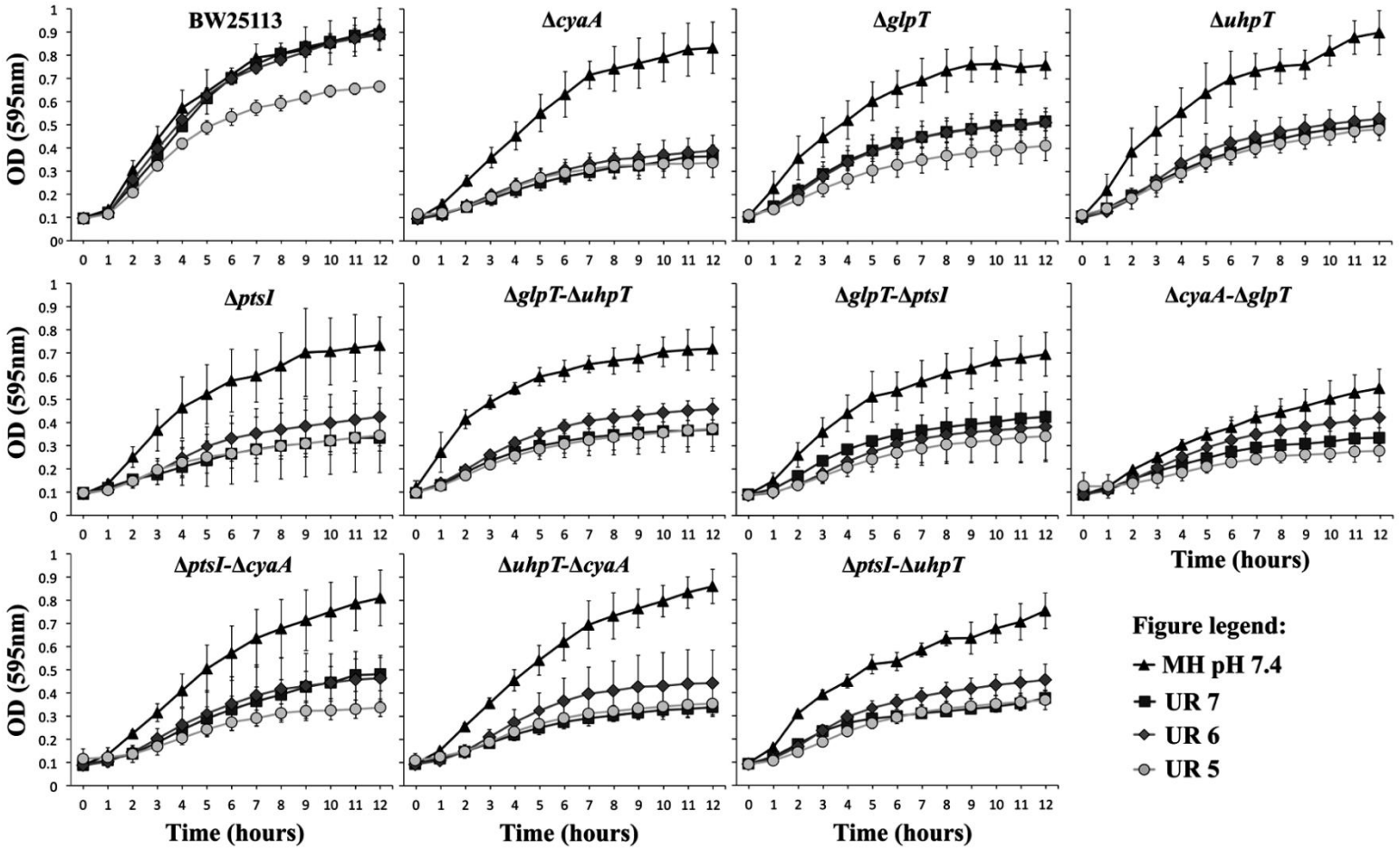

Figure 13: Growth curves of *E. coli* BW25113 and ten isogenic strains with deletions in genes related with FOS-resistance, growing in MH and urine at different pH values. Error bars represent standard errors of the means of results from at least ten replicates. MH, Mueller-Hinton; UR, urine; OD, optical density.