Convergent phenotypic evolution towards fosfomycin collateral sensitivity of *Pseudomonas aeruginosa* antibiotic-resistant mutants

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**Summary**

The rise of antibiotic resistance and the reduced amount of novel antibiotics support the need of developing novel strategies to fight infections, based on improving the use of the antibiotics we already have. Collateral sensitivity is an evolutionary trade-off associated with the acquisition of antibiotic resistance that can be exploited to tackle this relevant health problem. However, different works have shown that patterns of collateral sensitivity are not always conserved, thus precluding the exploitation of this evolutionary trade-off to fight infections. In this work, we identify a robust pattern of collateral sensitivity to fosfomycin in *Pseudomonas aeruginosa* antibiotic-resistant mutants, selected by antibiotics belonging to different structural families. We characterize the underlying mechanism of the collateral sensitivity observed, which is a reduced expression of the genes encoding the peptidoglycan-recycling pathway, which preserves the peptidoglycan synthesis in situations where its de novo synthesis is blocked, and a reduced expression of *fosA*, encoding a fosfomycin-inactivating enzyme. We propose that the identification of robust collateral sensitivity patterns, as well as the understanding of the molecular mechanisms behind these phenotypes, would provide valuable information to design evolution-based strategies to treat bacterial infections.

**Introduction**

Infections due to multidrug-resistant (MDR) bacteria, with limited therapeutic options, constitute an increasing concern for human health. Among them, *Pseudomonas aeruginosa* infections entail a clinical problem because of the low susceptibility of this microorganism to several antibiotics, its disruptive virulence mechanisms and its capacity to produce nosocomial infections, as well as chronic infections in patients with cystic fibrosis (CF) or chronic obstructive pulmonary disease (Martínez-Solano *et al*., 2008; Tummler *et al*., 2014; Talwalkar and Murray, 2016). The problem is aggravated by the increasing prevalence of infections due to *P. aeruginosa* antibiotic-resistant strains. In a situation where the amount of novel antibiotics introduced for therapy is low, novel therapeutic strategies are needed. Those based on the knowledge of the evolution of antibiotic resistance (AR) are particularly interesting to specifically tackle this relevant health problem.

One promising therapeutic strategy for specifically counteract AR would be the exploitation of trade-offs of AR evolution, such as collateral sensitivity. This phenomenon, firstly described in the fifties (Szybalski and Bryson, 1952), implies that the acquisition of AR to a given antimicrobial may lead to increased susceptibility to a second drug (Pal *et al*., 2015; Podnecky *et al*., 2018; Nichol *et al*., 2019). Some works have explored the possibility of exploiting collateral sensitivity in the aim of implementing therapeutic strategies that could reduce the chances for selecting AR (Pal *et al*., 2015; Baym *et al*., 2016), such as alternation of drug pairs (Imamovic and Sommer, 2013; Kim *et al*., 2014; Imamovic *et al*., 2018) or combinatory therapy (Munck *et al*., 2014; Barbosa *et al*., 2018; Jahn *et al*., 2021). Nevertheless, these approaches still have some drawbacks. As it could be expected, collateral sensitivity depends on the antibiotic used for selection and on the mechanisms involved in the acquisition of resistance (Imamovic and Sommer, 2013; Lazar *et al*., 2013; Barbosa *et al*., 2017; Podnecky *et al*., 2018; Nichol *et al*., 2019; Kavanaugh *et al*., 2020). In addition, different works have shown that the genetic
background may determine not only the evolutionary pathways towards AR but also the collateral sensitivity patterns acquired, indicating that historical contingency modulates AR evolution and the associated trade-offs (Baquero, 2013; Jochumsen et al., 2016; Hernando-Amado et al., 2019; Nichol et al., 2019). This implicates that, unless robust phenotypic patterns are found (Imamovic et al., 2018), the exploitation of collateral sensitivity for tackling AR may be compromised. In addition, the fact that the molecular mechanisms responsible for collateral sensitivity are still scarcely understood further compromises the possibility of taking advantage of this evolutionary trade-off to manage bacterial infections (Pal et al., 2015).

In previous works, we observed that all the P. aeruginosa PA14 populations that were submitted to adaptive laboratory evolution (ALE) in the presence of different antibiotics, namely ceftazidime, tobramycin or tigecycline (Sanz-Garcia et al., 2018a,2018b), presented collateral sensitivity to fosfomycin. This result is remarkable given that it provides a case of phenotypic convergence associated with the acquisition of resistance to antibiotics from different structural families, which suggests that fosfomycin could be alternated or used simultaneously with any of the three mentioned drugs.

Fosfomycin is an antibiotic that inhibits cell wall synthesis by targeting the enzyme MurA (UDP-N-acetylglucosamine 1-carboxyvinyltransferase), which catalyses the initial step of the peptidoglycan biosynthesis of the bacterial cell wall (Kahan et al., 1974). The major mechanism of fosfomycin resistance described for P. aeruginosa is the loss-of-function of glpT (Castaneda-Garcia et al., 2009), which encodes the fosfomycin transporter of this bacterium (Winkler, 1973). In addition, an increased expression of the gene encoding the fosfomycin target enzyme MurA, as well as the acquisition of mutations on it, has been described to increase fosfomycin resistance in other species, such as Mycobacterium tuberculosis, Borrelia burgdorferi or Chlamydia sp. (Venkateswaran and Wu, 1972; De Smet et al., 1999; McCoy et al., 2003; Jiang et al., 2011; Couce et al., 2012). Besides that, the expression of fosA, which encodes a fosfomycin-inactivating enzyme (De Groote et al., 2011; Silver, 2017), together with the activity of an alternative peptidoglycan-recycling pathway (Borisova et al., 2014; Fumeaux and Bernhardt, 2017; Hamou-Segarra et al., 2017), able to bypass MurA in the conversion of cell wall turnover products (Gisin et al., 2013), also contributes to the intrinsic resistance to fosfomycin of P. aeruginosa (Figure 1). Regarding the latter, the deletion of genes belonging to the peptidoglycan-recycling pathway has shown to increase susceptibility to fosfomycin in this bacterial species (Borisova et al., 2014).

Fosfomycin, alone or in combination with other antibiotics, as colistin, gentamicin, tigecycline, aztreonam, cefotolozane/tazobactam or piperacillin/tazobactam, is a valuable alternative for the treatment of difficult-to-treat infections produced by Gram-negative bacteria, such as carbapenemase-producing Enterobacteriaceae or MDR P. aeruginosa (Falagas et al., 2008a; Michalopoulos et al., 2010; Michalopoulos et al., 2011; Pontikis et al., 2014; Cuba et al., 2020). In addition, the co-administration of fosfomycin with ceftazidime-avibactam or tobramycin has been described to be synergistic against MDR P. aeruginosa strains (Papp-Wallace et al., 2019), as well as against CF P. aeruginosa biofilms (Diez-Aguilar et al., 2018). Furthermore, collateral sensitivity to fosfomycin is associated with the acquisition of resistance during ALE experiments in the presence of ceftazidime (alone or in combination with avibactam) or tobramycin (Sanz-Garcia et al., 2018a,2018b), a feature that has not been analysed in detail yet.

As above stated, it is quite uncommon to find conserved collateral sensitivity patterns (Barbosa et al., 2017), even when replicated populations from a single genetic background are treated with the same drug, which limits the exploitation of this evolutionary trade-off, although some particular examples have been described so far (Hernando-Amado et al., 2020; Roehmhold et al., 2020). For instance, we recently described that tobramycin collateral sensitivity associated with ceftazidime resistance acquisition was conserved in different genetic backgrounds. In this study, resistance acquisition was mediated by a conserved mechanism consisting in chromosomal deletions including several genes among which we found the aminoglycoside extruding efflux pump encoding genes mpxXY (Hernando-Amado et al., 2020). This robust collateral sensitivity phenotype was then the consequence of the parallel evolution towards ceftazidime resistance that, besides leading to such resistance, rendered the associated loss of a tobramycin intrinsic resistance determinant in all analysed lineages. Further, we observed that ceftazidime resistance acquisition also led to a conserved collateral sensitivity to fosfomycin, although the molecular mechanism implicated in this phenotype was not analysed in this previous work. In the present work, we found that, besides the mentioned collateral sensitivity to fosfomycin associated with the use of ceftazidime, this phenotype also emerges upon the selection of tobramycin or tigecycline-resistant mutants. Common patterns of collateral sensitivity of mutants selected in presence of different antibiotics have been rarely reported, so that we analyse in the current work the molecular mechanism behind this phenotype. We have found that this is the result of parallel expression profiles in the mutants resistant to each of the mentioned drugs. Therefore, we suggest that
fosfomycin, either alone or in combination with these antimicrobials, could be used to treat *P. aeruginosa* infections.

**Results**

*Experimental evolution of P. aeruginosa in presence of different antibiotics leads to convergent collateral sensitivity to fosfomycin*

The evolutionary pathways of *P. aeruginosa* PA14 in the presence of tobramycin, tigecycline or ceftazidime were previously determined after 30 days of ALE assays (Sanz-Garcia et al., 2018a, 2018b). Interestingly, all the resistant populations to tobramycin (four replicates), tigecycline (four replicates) or ceftazidime (four replicates) presented collateral sensitivity to fosfomycin, while control populations (evolved in the absence of antibiotics) did not show this phenotype (Sanz-Garcia et al., 2018a, 2018b). To determine if collateral sensitivity to fosfomycin of these populations was acquired at the initial step of the ALE experiments, the Minimal Inhibitory Concentration (MIC) of fosfomycin was determined, at day 5 of evolution, in a representative population resistant to either tigecycline, tobramycin or ceftazidime (hereafter dubbed as PpTgc5d, PpTob5d and PpCaz5d, respectively; see Table 1) (Table 2). At fifth day of evolution, fosfomycin MIC decreased from 32 µg ml⁻¹ in the PA14 wild-type strain to 4 µg ml⁻¹ in PpTgc5d and PpTob5d populations and to 2 µg ml⁻¹ in PpCaz5d population (Table 2). In addition, no changes in fosfomycin MIC were observed at 5 days of ALE in the control populations (PpControlTgc5d-Tob5d and PpControlCaz5d; see Table 1), indicating that collateral sensitivity to fosfomycin was early selected by the three antibiotics (Table 2). The MIC of the antibiotic used as selective pressure in each evolved population was also measured to determine the level of resistance acquired on the fifth day of each ALE, compared to the control populations (Table 2). In addition, since there could be heterogeneity in fosfomycin susceptibility within each of the three populations tested, ten individual clones from each population were isolated and their fosfomycin susceptibility was measured.

Fig. 1. Schematic representation of the *Pseudomonas aeruginosa* fosfomycin resistome. Fosfomycin (FOF) enters the cell through GlpT transporter (Winkler, 1973) and, once inside the cell, fosfomycin could be inactivated by FosA. A reduced expression of *fosA* increases the intracellular amount of active fosfomycin. Active fosfomycin blocks the de novo peptidoglycan synthesis by the inhibition of MurA (Kahan et al., 1974). In this situation, the synthesis of the peptidoglycan may be maintained by the peptidoglycan-recycling pathway, whose loss-of-function leads to hyper-susceptibility to fosfomycin (Borisova et al., 2014). Data shown in this work propose that a reduced expression of both, genes belonging to the peptidoglycan-recycling pathway and *fosA*, is at the molecular basis of collateral sensitivity to fosfomycin observed in different antibiotic-resistant mutants.
measured by disc diffusion. No differences were observed, neither among the clones within a population, nor with respect to the populations to which they belong. Therefore, a representative clone from each population (hereafter dubbed as Tgc5d, Tob5d and Caz5d; see Table 1) was chosen. To ascertain if the fitness of Tgc5d, Tob5d and Caz5d mutants could be affected respect to the wild-type strain, growth curves in LB were performed. Growth defects (growth rate and/or final optical density) were not detected in the resistant mutants. Further, a growth increase was observed in Tob5d mutant (Figure 2).

Whole-genome sequencing of the Tgc5d, Tob5d and Caz5d mutants

In order to elucidate the genetic causes associated with collateral sensitivity to fosfomycin of the studied clones, their genomes were sequenced. The genetic variations found in these clones are described in Table 3. The Tgc5d clone contains two different genetic variations in the gene coding for NfxB, the negative regulator of the expression of mexCD-oprJ \cite{Poole1996, Pursell2013}, which encodes an efflux pump that extrudes quinolones, β-lactams and chloramphenicol (De

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### Table 1. Bacterial strains and plasmids used in this work.

| Bacterial strains   | Description                                      | Reference/origin          |
|---------------------|--------------------------------------------------|---------------------------|
| *Escherichia coli*  |                                                  |                           |
| DH5α                | Host strain used for the maintenance of cloning plasmids | Laboratory collection    |
| OmniMAX™            | Fosfomycin susceptible strain used for the estimation of the intracellular content of fosfomycin of the studied antibiotic-resistant mutants | Invitrogen               |
| **Pseudomonas aeruginosa** |                                              |                           |
| PA14                | Wild-type strain of *P. aeruginosa*              | Laboratory collection    |
| PpTgc5d             | Population evolved 5 days in tigecycline         | Sanz-García *et al.* (2018b) |
| PpTob5d             | Population evolved 5 days in tobramycin          |                           |
| PpControlTgc5d-Tob5d| Control population evolved 5 days in the absence of antibiotics | Sanz-García *et al.* (2018a) |
| PpCaz5d             | Population evolved 5 days in ceftazidime         |                           |
| PpControlCaz5d      | Control population evolved 5 days in the absence of antibiotics |                           |
| Tgc5d               | Isolated clone from PpTgc5d                      | This study                |
| Tob5d               | Isolated clone from PpTob5d                      | This study                |
| Caz5d               | Isolated clone from PpCaz5d                      | This study                |
| PLM011              | Wild-type strain of PA14 carrying pPLM001 vector | This study                |
| PLM021              | Tgc5d clone carrying pPLM001 vector              | This study                |
| PLM031              | Tob5d clone carrying pPLM001 vector              | This study                |
| PLM041              | Caz5d clone carrying pPLM001 vector              | This study                |
| PLM010              | Wild-type strain of PA14 carrying pSEVA234 vector| This study                |
| PLM020              | Tgc5d clone carrying pSEVA234 vector             | This study                |
| PLM030              | Tob5d clone carrying pSEVA234 vector             | This study                |
| PLM040              | Caz5d clone carrying pSEVA234 vector             | This study                |
| fosA4::Tn           | *P. aeruginosa* PA14 transposon insertion mutant in fosA | Liberati *et al.* (2006) |
| agmK::Tn            | *P. aeruginosa* PA14 transposon insertion mutant in agmK | Liberati *et al.* (2006) |
| murU::Tn            | *P. aeruginosa* PA14 transposon insertion mutant in murU | Liberati *et al.* (2006) |
| glpT::Tn            | *P. aeruginosa* PA14 transposon insertion mutant in glpT | Liberati *et al.* (2006) |
| orfN*               | *P. aeruginosa* PA14 mutant in orfN | Hernando-Amado *et al.* (2019) |
| **Plasmids**        |                                                  |                           |
| pGEM-T Easy         | Commercial plasmid used for cloning optimization of PCR products. AmpR | Promega                  |
| pSEVA234            | Plasmid used for overexpression of genes with a strong promoter controlled by IPTG. KmR | Donated by Victor de Lorenzo's laboratory GenBank: KC847292.2 |
| pPLM001             | Plasmid pSEVA234 used for overexpression of fosA gene with a strong promoter controlled by IPTG. KmR | This study |

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### Table 2. MIC values (µg ml⁻¹) of *P. aeruginosa* populations and strains used in this study.

|           | TGC | TOB | CAZ | FOF |
|-----------|-----|-----|-----|-----|
| PA14      | 2   | 0.75| 0.75| 32  |
| PpControlTgc5d-Tob5d | 2   | 0.75| 0.75| 32  |
| PpControlCaz5d | 2   | 0.75| 0.75| 32  |
| PpTgc5d   | 32  | 0.75| 1   | 4   |
| PpTob5d   | 4   | 4   | 1.5 | 4   |
| PpCaz5d   | 2   | 0.38| 8   | 2   |
| Tgc5d     | 32  | 0.75| 1   | 4   |
| Tob5d     | 48  | 4   | 1.5 | 4   |
| Caz5d     | 2   | 0.38| 8   | 2   |
| orfN*     | 12  | 3   | 3   | 8   |

CAZ, ceftazidime; FOF, fosfomycin; TGC, tigecycline; TOB, tobramycin.
MIC values of the antibiotics used as selective pressure during ALE experiments are highlighted in bold.

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be encountered in clinical strains (Jalal and Wretling, 1998; Mayer-Hamblett et al., 2014; Bolard et al., 2018), a feature supporting that our ALE-derived results have clinical implications.

Importantly, a genetic change in orfN, which encodes a putative glycosyl transferase needed for the glycosylation of type A flagellins (Schirm et al., 2004), was shared by the three resistant clones. While this gene may be contributing to AR in the three clones, since orfN mutations have been selected during ALE experiments in presence of ciprofloxacin, ceftazidime, tobramycin, aztreonam or tigecycline (Wong et al., 2012; Jorth et al., 2017; Sanz-Garcia et al., 2018a, 2018b; Hernando-Amado et al., 2019), its role in susceptibility to fosfomycin is unknown. Therefore, susceptibility to fosfomycin of an orfN mutant (orfN*) (Hernando-Amado et al., 2019) was determined. As shown in Table 2, the MIC of orfN* (8 µg ml⁻¹) was lower than that of the wild-type strain (32 µg ml⁻¹). However, this reduction did not reach the levels of those observed in the hyper-susceptible populations (up to 2 µg ml⁻¹), indicating that the observed collateral sensitivity was not just due to the mutation in orfN. However, no other mutations that could explain collateral sensitivity to fosfomycin observed in the three mutants were found. Neither in fosA, which encodes a fosfomycin-inactivating enzyme (Silver, 2017), nor in annK, mupP, agmK and murU, which encode the enzymes of the peptidoglycan-recycling pathway (Borisova et al., 2014; Fumeaux and Bernhardt, 2017) or nagZ, the gene upstream this pathway (Borisova et al., 2014). As might be expected, given that the mutants present an increased susceptibility to fosfomycin, no mutations were detected in the gene encoding GlpT, the only fosfomycin transporter described so far in P. aeruginosa (Winkler, 1973; Castaneda-Garcia et al., 2009), neither in murA, that encodes the fosfomycin target (Silver, 2017).

### Table 3. Genetic changes detected in the Tgc5d, Tob5d and Caz5d clones.

| Gene    | Genetic change | Localization | Aa change | Clone          |
|---------|----------------|--------------|-----------|----------------|
| orfN    | Del G          | 139          | Val50fs   | Tgc5d, Tob5d   |
| Ins G   | Del            | 138-139      | Val50fs   | Caz5d          |
| nfxB    | Del GGGAGGC    | 231-236      | Glu78Ala79del | Tgc5d           |
| Ins     | GGGAGGC        | 233-236      | Lys132delinsArg | ArgGln         |
| fusA    | G→C            | 1783         | Ala595Pro | Tob5d          |
|         | Del            | 299,658 kb   | 3200274   | Caz5d          |

a. Nucleotide location of the mutations, referred to the gene in which they are located, and the amino acid changes associated. Location of the large chromosomal deletion refers to the nucleotide position in P. aeruginosa UCBPP-PA14 reference chromosome (NC_008463.1). fs: frameshift. Del: deletion. Ins: insertion.

Kievit et al., 2001). The Tob5d clone presents a mutation in fusA, which encodes an elongation factor associated with aminoglycosides resistance (Wang et al., 2015; Feng et al., 2016; Bolard et al., 2018). A large chromosomal deletion of around 300 kb was detected in the Caz5d clone, containing the gene galU, whose inactivation increases ceftazidime resistance (Alvarez-Ortega et al., 2010; Sanz-Garcia et al., 2018a), which was previously described to be robustly acquired upon ceftazidime selective pressure in several genetic backgrounds (Hernando-Amado et al., 2020). Genetic alterations detected in the studied clones, such as mutations in fusA or nfxB, or the detected large deletions, have been described to

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are known to be associated with intrinsic resistance of *P. aeruginosa* to fosfomycin presented a reduced expression in the studied mutants (Table 4; Data S1). Among them, *fosA* and the genes encoding the peptidoglycan-recycling pathway (*agmK, murU, anmK, nagZ* and *mupP*) can be highlighted. In addition, despite the observed hyper-susceptibility to fosfomycin of the three mutants, the expression of *glpT*, that encodes the only known fosfomycin transporter in *P. aeruginosa*, was reduced in Tgc5d and Tob5d, but not in Caz5d (Table 4; Data S1).

The expression of the mentioned genes was analysed by quantitative reverse transcription PCR (qRT-PCR) (Figure 3), thus validating the RNA-seq analysis. A significant reduction of the expression of *fosA*, up to the half in Tgc5d or Tob5d and a quarter in Caz5d mutant, and of some of the genes encoding the peptidoglycan-recycling pathway enzymes, was observed (Figure 3). It

![Fig. 3. Expression level of genes encoding intrinsic fosfomycin resistance determinants in Tgc5d, Tob5d and Caz5d mutants. Fold changes of Tgc5d (blue), Tob5d (red) and Caz5d (green) were estimated regarding the expression of the PA14 wild-type strain by qRT-PCR. Error bars indicate standard deviations of the results from three biological replicates. Statistically significant differences regarding PA14 were calculated with *t*-test for paired samples assuming equal variances: *P* < 0.05; **P* < 0.005; ***P* < 0.0005.](image-url)

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is known that both, the inactivation of genes encoding the peptidoglycan-recycling pathway (Borisova et al., 2014), as well as the inhibition of FosA (Ito et al., 2017), increase P. aeruginosa susceptibility to fosfomycin. Hence, the reduced expression of these genes may be the reason for the fosfomycin collateral sensitivity of the analysed antibiotic-resistant mutants.

To determine if the expression changes detected in fosA and the genes that encode the peptidoglycan-recycling pathway could be caused by the orfN* mutation present in the three hyper-susceptible mutants, a transcriptomic analysis was performed for orfN* (Data S1). Although this mutant presents a reduced MIC to fosfomycin compared to the wild-type, no alteration in the expression of the genes encoding the enzymes of the peptidoglycan-recycling pathway and a slight reduction of fosA expression (just in the 0.75 threshold) was detected (Data S1). The reduction of fosA expression may explain the increased fosfomycin susceptibility of the orfN* mutant, as well as part of the collateral sensitivity to fosfomycin observed in the three mutants. However, since this phenotype is weaker in orfN* than in the analysed resistant mutants (Table 2) and, in addition, orfN* does not present alterations in the expression of genes encoding the peptidoglycan-recycling pathway, we hypothesize that the parallel expression profiles observed in the three mutants might result from an unknown epistatic interaction between orfN* and the AR mutations present in the hyper-susceptible mutants. In fact, epistatic interactions in which orfN* participates have been previously described (Hernando-Amado et al., 2019).

Regarding the expression of genes that might contribute to the AR phenotype of the Tgc5d, Tob5d and Caz5d mutants, the RNA-seq analysis showed that the expression of mexCD-oprJ in the Tgc5d clone is increased by 500-fold respect to the wild-type strain (Data S1), possibly contributing to tigecycline resistance (Sanz-Garcia et al., 2018b). This may be explained by the genetic modifications present in nfxB (Table 3), which encodes the local mexCD-oprJ repressor (Purssell and Poole, 2013). The genes cipB, hslV and ibpA are overexpressed in the Tob5d mutant (Data S1). Since P. aeruginosa knockout mutants in these genes are tobramycin hyper-susceptible (Wu et al., 2015), their overexpression may be contributing also to tobramycin resistance in the Tob5d mutant. In agreement with the reduced susceptibility to ceftazidime of clone Caz5d, the β-lactamase encoding gene ampC is overexpressed by more than 100-fold respect to the wild-type strain (Data S1). In addition, as expected, no expression of galU, included in the large chromosomal deletion of this mutant, was detected, which may also be contributing to ceftazidime resistance (Alvarez-Ortega et al., 2010).

Finally, common expression changes of genes that encode catabolic-related enzymes were observed for every fosfomycin hyper-susceptible mutant (Data S1). Regarding the carbohydrate metabolism, we observed an increased expression of genes encoding enzymes of the glycolysis pathway (gapA and gldA), the phosphate pentose pathway (zwf and pgI), the Entner Doudoroff pathway (edd), the Krebs cycle (aconA and gltA) and the glyoxylic cycle (gltB and PA14_30050), as well as of the glucose transporters PA14_22980, PA14_22990 and PA14_23000, yet a decreased expression of lactate dehydrogenase encoding genes (lldA and lidD). Moreover, we also found increased expression of some genes that encode amino acid and fatty acid degradation enzymes, such as the bkd operon (lpdv, bkdB, bkdA1 and bkdA2), which controls the assimilation of branched amino acids (Corona et al., 2018), or fadE, that encodes an enzyme that catalyses one reaction in the β-oxidation pathway (Zarzycki-Siek et al., 2013). As far as we know, a correlation between an increased expression of the genes encoding for catabolic-related enzymes, which are differentially expressed in the Tgc5d-, Tob5d- and Caz5d-resistant mutants, and collateral sensitivity to fosfomycin, has not been described.

The reduced expression of fosA and the peptidoglycan-recycling pathway genes are jointly responsible for collateral sensitivity to fosfomycin of Tgc5d, Tob5d and Caz5d mutants

The transcriptomic analysis of the Tgc5d, Tob5d and Caz5d mutants showed a reduced expression of fosA and of the genes encoding the peptidoglycan-recycling pathway that could explain their collateral sensitivity to fosfomycin. However, we also observed that two of the mutants (Tgc5d and Tob5d) presented a reduced expression of glpT, which could render an impaired accumulation of intracellular fosfomycin. To find out if the altered expression of these genes could modify the amount of active fosfomycin in these mutants, the intracellular concentration of this antibiotic was measured and compared with that of the wild-type strain. Insertion mutants in the genes glpT and fosA, from a non-redundant transposon insertion library of P. aeruginosa PA14 (Liberati et al., 2006), were used as controls for low and high amount of intracellular fosfomycin, respectively. As shown in Figure 4, the intracellular concentration of fosfomycin in the mutants Tgc5d, Tob5d and Caz5d, after incubation with this antibiotic (see Experimental procedures), was threefold the one of the wild-type strain, but lower than in the fosA insertion mutant, whose intracellular concentration of fosfomycin was eightfold the one of the wild-type strain. In addition, the intracellular accumulation of fosfomycin in the orfN* mutant was also

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measured, since it presents a reduced expression of \textit{fosA} compared to the wild-type strain. Similar levels to those ones measured in Tgc5d, Tob5d and Caz5d mutants were detected for \textit{orfN*} (Figure 4), indicating that the mutation in this gene may be, at least in part, responsible for the reduced expression of \textit{fosA} and the augmented fosfomycin accumulation in these mutants. Nevertheless, the MIC to fosfomycin of \textit{orfN*} is higher than those ones of Tgc5d, Tob5d and Caz5d mutants (Table 2). As stated above, these results support that besides the increased accumulation of intracellular fosfomycin, the reduced expression of the peptidoglycan-recycling pathway genes is contributing to fosfomycin hyper-susceptibility of the studied mutants.

In order to estimate the contribution of FosA and of the peptidoglycan-recycling enzymes to the intrinsic resistance to fosfomycin of \textit{P. aeruginosa}, the fosfomycin MIC of the \textit{fosA}, \textit{agmK} and \textit{murU} insertion mutants, obtained from the mentioned non-redundant transposon insertion library of \textit{P. aeruginosa} PA14 (Libreri et al., 2006), was determined and compared with the ones of the Tgc5d, Tob5d and Caz5d mutants (Table 5). The transposon insertion mutants in either \textit{agmK} or \textit{murU} presented a fosfomycin MIC of 8 \(\mu\text{g ml}^{-1}\) (a quarter of the one of the wild-type parental strain), while the insertion mutant in \textit{fosA} presented a fosfomycin MIC of 2 \(\mu\text{g ml}^{-1}\) (Table 5). Fosfomycin MICs for Tgc5d and Tob5d were close to the one of the \textit{fosA} insertion mutant, being exactly the same in the case of Caz5d (Table 5), while the intracellular concentration of fosfomycin in the mutants Tgc5d, Tob5d and Caz5d was much lower than in the \textit{fosA} insertion mutant (Figure 4). These results suggest that collateral sensitivity to fosfomycin may not be just a result of the observed reduced expression of \textit{fosA}. Indeed, if the increased intracellular concentration of fosfomycin was the only element involved in the collateral sensitivity of the studied mutants, they should present fosfomycin MICs higher than the \textit{fosA} mutant. Consequently, other factors, such as the mentioned reduced expression of the genes belonging to the peptidoglycan-recycling pathway, must be contributing to collateral sensitivity to fosfomycin of the studied mutants.

To further analyse the different contribution of the peptidoglycan-recycling pathway and of FosA to \textit{P. aeruginosa} fosfomycin susceptibility, the fosfomycin MICs of the \textit{fosA}, \textit{agmK} and \textit{murU} insertion mutants, as well as of the Tgc5d, Tob5d and Caz5d mutants, were determined in presence of phosphonoformate (Table 5), an

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**Fig. 4.** Active intracellular fosfomycin accumulation in Tgc5d, Tob5d, Caz5d and \textit{orfN*} mutants. The amount of active intracellular fosfomycin is represented as \(\mu\text{g}\) of active fosfomycin in \(10^9\) cells, after 1 h of incubation with 2 mg ml\(^{-1}\) of the antibiotic. The resulting values were estimated regarding the halo produced in an \textit{E. coli} OmniMAX\textsuperscript{TM} seeded plate, by a disc soaked with the intracellular content. \textit{P. aeruginosa} PA14 \textit{glpT} and \textit{fosA} transposon insertion (Tn) mutants were used as controls of low and high amount of intracellular fosfomycin, respectively. Error bars indicate standard deviations of the results from three biological replicates. Statistically significant differences were calculated with \(t\)-test for paired samples assuming equal variances: \(*P < 0.05.\)

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**Table 5.** Fosfomycin MIC values (\(\mu\text{g ml}^{-1}\)) in presence or absence of an inhibitor of FosA of transposon insertion mutants in genes encoding intrinsic fosfomycin resistance determinants and of Tgc5d, Tob5d and Caz5d mutants.

|         | \textit{fosA}: Tn | \textit{agmK}: Tn | \textit{murU}: Tn | Tgc5d | Tob5d | Caz5d |
|---------|------------------|------------------|------------------|-------|-------|-------|
| MHA     | 32               | 8                | 8                | 4     | 4     | 2     |
| PPF     | 8                | 2                | 1.5              | 2     | 2     | 1     |

PPF, FosA inhibitor phosphonoformate (500 \(\mu\text{M}).\)

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inhibitor of FosA (Ito et al., 2017). As expected, this FosA inhibitor does not affect the fosfomycin MIC of the fosA insertion mutant (Table 5). However, its presence reduces fosfomycin MICs in the agmK and murU insertion mutants to levels close to those of the fosA insertion mutant (Table 5). Importantly, fosfomycin MIC of the Tgc5d, Tob5d and Caz5d mutants was even lower in the presence of the inhibitor of FosA, up to 1.5 µg ml⁻¹, while inhibition of FosA in the wild-type strain reduced the MIC just to 8 µg ml⁻¹ (Table 5). These results indicate that intrinsic fosfomycin resistance in P. aeruginosa depends on the activity of both, FosA and the peptidoglycan-recycling pathway and that the observed reduced expression of these genes in the mutants Tgc5d, Tob5d and Caz5d is jointly contributing to their collateral sensitivity to fosfomycin.

Finally, fosA was overexpressed in Tgc5d, Tob5d and Caz5d mutants and the wild-type strain and the intracellular concentration of fosfomycin was measured in all of them. As shown in Figure 5, fosA overexpression reduces the accumulation of fosfomycin in the studied mutants, as well as in the wild-type strain. Consistent with these findings, fosfomycin MIC increased above the limit of detection by E-test (> 1024 µg ml⁻¹) in the mutants and in the wild-type strain when fosA is overexpressed. This result further supports that the collateral sensitivity to fosfomycin observed in the mutants is, at least in part, a consequence of a reduced fosA expression. However, the fact that the inhibition of FosA leads to higher susceptibility in the mutants than in the wild-type strain (Table 5), supports that the reduced activity of the peptidoglycan-recycling pathway in the mutants, which impedes the alternative preservation of the cell wall in situations where MurA does not work, is also contributing to the convergent phenotype of the three analysed mutants.

The frequency of emergence of fosfomycin-resistant mutants is lower in Tgc5d, Tob5d and Caz5d mutants than in the wild-type strain

One of the main drawbacks traditionally argued against the clinical use of fosfomycin is the high frequency of resistant mutants that are selected by this drug in vitro, particularly in the case of P. aeruginosa (Rodriguez-Rojas et al., 2010). However, acquisition of resistance to fosfomycin is rarely observed in vivo during therapy of urinary tract infections (Silver, 2017), which allows the use of fosfomycin for treating, among others, infections caused by MDR pathogens (Falagas et al., 2008b; Falagas et al., 2009; Falagas et al., 2010). In addition, it is known that the ‘apparent mutation frequency’ for one...
antibiotic depends on the concentration of the antibiotic used for selection (Garcia-Leon et al., 2014), a feature described in the case of fosfomycin (Demir and Buyukguclu, 2017; Falagas et al., 2019). To ascertain if the studied resistant mutants, presenting collateral sensitivity to fosfomycin, may also present a decreased fosfomycin resistance mutant frequency (at least in vitro), the emergence of fosfomycin-resistant mutants at different concentrations of fosfomycin (32, 64, 128 and 256 \( \mu \text{g ml}^{-1} \)) was measured in the three mutants and the wild-type strain. As shown in Figure 6, the fosfomycin-mutant frequency decreased in the three mutants compared with the wild-type strain in a concentration-dependent way. Indeed, for the highest concentration analysed, fosfomycin-mutant frequency was reduced by up to 19.6-, 9.4- and 5.5-fold in Tgc5d, Tob5d and Caz5d, respectively.

**Discussion**

The analysis of collateral sensitivity networks has been proposed as a good approach for implementing strategies in the use of anticancer (Pluchino et al., 2012) and antimicrobial (Imamovic et al., 2018; Podnecky et al., 2018) compounds, which would reduce the burden of resistance (Lazar et al., 2013; Hancock, 2014; Baym et al., 2016). Nevertheless, with few exceptions (Imamovic et al., 2018), collateral sensitivity depends on the antibiotic used for selection and the resistance mechanism involved. Further, in most cases so far studied, collateral sensitivity is not conserved across different genetic backgrounds (Lazar et al., 2013; Lazar et al., 2014; Barbosa et al., 2017; Podnecky et al., 2018) and the molecular bases of collateral sensitivity remain obscure (Pal et al., 2015). This stochasticity in the collateral sensitivity networks preclude a general use of this evolutionary trade-off, since it can be exploited just when robust collateral sensitivity patterns are found.

We have recently identified a robust collateral sensitivity pattern to tobramycin in *P. aeruginosa* mutants selected in presence of ceftazidime (Hernando-Amado et al., 2020). Despite the original strains submitted to evolution presented a different genetic background, the genetic event leading to ceftazidime resistance were the same; the deletion of a chromosomal region that, among several genes, contains the intrinsic tobramycin resistance determinant *mexXY* (Hernando-Amado et al., 2020). Collateral sensitivity in this case was a consequence of parallel evolution of different bacterial lineages submitted to the same selective pressure.

![Fig. 6. Fosfomycin-resistant mutant frequency of wild-type *P. aeruginosa* PA14 and Tgc5d-, Tob5d- and Caz5d-resistant mutants. The frequency of fosfomycin-resistant mutants in the wild-type PA14 (grey) and the mutants Tgc5d (blue), Tob5d (red) and Caz5d (green) was estimated in the presence of different concentrations of fosfomycin. In the four genomic backgrounds analysed, it resulted to be inversely proportional to the concentration used for selection. As shown, the observed mutant frequency was lower in all the analysed mutants than in the wild-type strain. Error bars indicate standard deviations of the results from three biological replicates. Statistically significant differences regarding PA14 were calculated with *t*-test for paired samples assuming equal variances: \(*P < 0.05; \,**P < 0.005; \,\,**\,*P < 0.0005.*](image)
In the current work, the situation is more complex, since the different bacterial lineages are submitted to different selective pressures. Despite this situation, we identified a convergent, robust phenotype of collateral sensitivity displayed by different antibiotic-resistant mutants of *P. aeruginosa* previously selected *in vitro* along ALE experiments in the presence of different antibiotics: tobramycin, tigecycline or ceftazidime. Differing to the situation analysed in (Hernando-Amado *et al.*, 2020), this is an example of phenotypic convergence as a response to different selective pressures. Moreover, we identified the underlying molecular mechanisms of collateral sensitivity to fosfomycin in the three antibiotic-resistant mutants: a reduced expression of the genes encoding the peptidoglycan-recycling pathway, that preserves the peptidoglycan synthesis in situations where its *de novo* synthesis is blocked, and a reduced expression of *fosA*.

The WGS analysis of the resistant clones showed that Tgc5d, Tob5d and Caz5d do not present mutations in any of the elements that have been described to play a role in intrinsic resistance to fosfomycin, but all of them acquired a mutation in *orfN*. The analysis of Tgc5d, Tob5d, Caz5d and *orfN*+ transcriptomes showed a reduced expression of *fosA* that could be responsible for the reduced amount of intracellular fosfomycin detected in all of them. However, Tgc5d, Tob5d and Caz5d mutants also presented a reduced expression of the genes encoding the peptidoglycan-recycling pathway and, therefore, an increased susceptibility to fosfomycin compared with *orfN*+. We hypothesize that the rewiring of the transcriptome caused by the combination of *orfN*+ and the AR mutations present in Tgc5d, Tob5d and Caz5d mutants is responsible for the parallel expression profiles of the three mutants. Although it is generally assumed that the effect of loss-of-function mutations in the global transcriptome may reflect the loss of specific molecular functions associated with the disrupted gene, it has recently been described that rewiring of the transcriptome upon deleterious gene inactivation is frequently non-specific and mimics stereotypic responses to external environmental changes (Kovács *et al.*, 2021). Additional experiments will be required to address these questions in the framework of the acquisition of AR and the associated collateral sensitivity patterns.

Although common expression profiles were detected for the three studied mutants regarding *fosA* and peptidoglycan-recycling enzymes encoding genes, this was not a general feature for their overall transcriptomes. Indeed, many differences in expression of other genes unrelated to fosfomycin susceptibility were detected. Among them, it seems that the genes for pyochelin biosynthesis and uptake are quite overexpressed in Tgc5d, Tob5d, but not in Caz5d, whereas QS dependent on PQS is downregulated in the latter. This indicates that the observed robust collateral sensitivity pattern is not a result of a common global physiological changes, but rather of the parallel expression changes in genes involved in fosfomycin resistance. This is a feature that may be expected having into consideration that mutations in a global regulator, an elongation factor or the loss of more than 250 genes occurs in Tgc5d, Tob5d or Caz5d mutants, respectively, and it is difficult that the effect of these disparate genetic alterations in bacterial physiology is overall the same. Actually, mutations in *ntxB* or *fusA* have been described to produce global changes in the transcriptome and proteome of *P. aeruginosa* (Stickland *et al.*, 2010; Maunders *et al.*, 2020). It is possible that the different transcriptomic changes observed in Tgc5d, Tob5d or Caz5d may alter their virulence, however, this possibility is beyond the purpose of the current work. Nevertheless, the observation that, despite the global transcriptomes of Tgc5d, Tob5d or Caz5d differ, common changes in the expression of fosfomycin intrinsic-resistant genes are found, reinforces the importance of having detected a convergent collateral sensitivity to fosfomycin associated with the down-regulation of the same genes after bacterial adaptation to different drugs.

Besides collateral sensitivity to fosfomycin, we also found that fosfomycin-mutant frequency is highly reduced in the studied mutants compared with the wild-type PA14. Previous work has shown that fosfomycin-resistant mutants of *P. aeruginosa* emerge at high frequency *in vitro* when model, antibiotic susceptible strains, are analysed (Rodriguez-Rojas *et al.*, 2010), although they are not so frequently detected *in vivo*, where antibiotic selective pressure is high (Silver, 2017). Despite previous concerns, this situation and the early selection of this phenotype (at fifth day of ALE experiments) allow the use of this antibiotic, alone or in combination, for treating different types of infections, including those caused by MDR pathogens (Falagas *et al.*, 2008b; Falagas *et al.*, 2009; Falagas *et al.*, 2010). Since we found that the studied resistant mutants present a robust collateral sensitivity to fosfomycin, together with a reduced fosfomycin-mutant frequency, it is tempting to speculate that these evolutionary trade-offs might contribute to the success of fosfomycin for inhibiting MDR *P. aeruginosa* strains. Altogether, the observations here reported and previously published information (Falagas *et al.*, 2008a; Falagas *et al.*, 2009; MacLeod *et al.*, 2012; Diez-Aguilar *et al.*, 2015; Keepers *et al.*, 2017; Diez-Aguilar *et al.*, 2018; Gopichand *et al.*, 2019; Papp-Walace *et al.*, 2019; Hernando-Amado *et al.*, 2020) point to fosfomycin as a valuable antibiotic to be introduced in sequential or combinatorial therapies against *P. aeruginosa* infections. According to the *in vitro* data here
discussed and the results previously obtained (Hernando-Amado et al., 2020), an alternation or combination of the antibiotics fosfomycin-tobramycin-ceftazidime could be particularly promising.

In agreement with previous statements (Imamovic et al., 2018; Hernando-Amado et al., 2020; Roehmchild et al., 2020), we propose that robust phenotypes of collateral sensitivity should be identified and exploited for treating bacterial infections. Particularly relevant will be finding phenotypic convergent patterns (Imamovic et al., 2018) as those described in the current study. However, the feasibility of rational broad-spectrum collateral sensitivity-based strategies will require a deeper knowledge of the molecular mechanisms that increase antibiotic susceptibility upon resistance acquisition to certain antibiotics. In this regard, here we describe that a reduced expression of both fosA and the genes encoding the peptidoglycan-recycling pathway are responsible for collateral sensitivity to fosfomycin in different antibiotic-resistant mutants. Another limitation of collateral sensitivity to be clinically applied is that resistance to antibiotics frequently occurs by the acquisition of AR genes mediated by plasmids. Nevertheless, a recent work has identified a case of collateral sensitivity robustness associated with the acquisition of the clinically important carbapenem resistance conjugative plasmid pOXA-48 (Herencias et al., 2021), which may open the possibility of exploiting the said trade-off even in the case of acquired genes. In summary, although extensive work is still required until evolution-based anti-infection approaches could be applied in clinics, we think that the results here presented and those already published provide enough evidence to support that the time has come to translate evolutionary knowledge into medical breakthrough to tackle AR.

**Experimental procedures**

**Culture conditions and determination of susceptibility to antibiotics**

Unless stated otherwise, all strains were grown in Lyso-geny Broth (LB) (Lennox, Pronadisa, Torrejón de Ardoz, Spain) at 250 rpm or in LB agar (1.5% agar), at 37°C. The *E. coli* strains carrying the pSEVA234-derived plasmid or pGEM-T Easy Vector (Promega, Madison, WI, USA) were grown in LB with 50 µg ml⁻¹ of kanamycin or 100 µg ml⁻¹ of ampicillin, respectively. Strains and plasmids used in this work are included in Table 1.

Antibiotic susceptibility was determined in Mueller-Hinton Agar (MHA, Pronadisa, Torrejón de Ardoz, Spain) at 37°C using MIC Test Strips (Liofilchem®, Roseto degli Abruzzi, Italy) following supplier’s instructions or by disc diffusion. MHA plates were supplemented with 500 µM of phosphonoformate (PPF) when needed.

**Whole-genome sequencing and bioinformatics analysis**

The genomic DNA of each isolated clone was extracted using the Gnome® DNA kit (MP Biomedicals, Solon, OH, USA). The DNA quality analysis and the whole-genome sequencing (WGS) were performed by Novogene Bioinformatics Technology (Tianjin, China). Libraries constructed were pair-end (2 × 350) and sequenced with an Illumina PE150 system. The average number of reads per sample represents a coverage > 200x. WGS data were analysed by using the CLC Genomics Workbench 12.0 software (Qiagen, Düsseldorf, Germany) by trimming genomic information and aligning the reads against the GenBank *P. aeruginosa* UCBPP-PA14 reference chromosome (NC_008463.1).

**RNA preparation, RNA-sequencing and qRT-PCR**

Overnight cultures of *P. aeruginosa* PA14, Tgc5d, Tob5d, Caz5d and orfN⁺ were used to inoculate 20 ml of LB medium to a final OD₆₀₀nm of 0.01 and were grown until exponential phase (OD₆₀₀nm = 0.6). Afterwards, centrifugation of 10 ml at 7000 rpm and 4°C, for 20 minutes, was performed.

The RNA extraction from the collected cells was performed as previously described in (Blanco et al., 2017). DNA was removed by treatment with Turbo DNA-free (Ambion), and the absence of DNA was checked by PCR using primers rpsL_Fw and rpsL_Rv (Table 6). From 10 µg of RNA, cDNA was obtained by using the High-Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA).

RNAs obtained from three independent cultures of each strain were pooled, and RNA-sequencing (RNA-seq) was performed at the Next Generation Sequencing Service of the Centre for Research in Agricultural Genomics by using paired end format lectures (2 × 75bp) in an Ion PGM™ Sequencer. Rockhopper (McClure et al., 2013) was used for analysing the results. Those genes with a level of expression below 15 Reads Per Kilobase Million (RPKM) in the wild-type strain and in the mutants were discarded for further analysis. The fold change threshold for the genes up- or downregulated in the mutants compared with the wild-type strain was settled at 1.5 and 0.75, respectively. RNA-seq data included in this work are deposited in GEO database with accession GSE153006.

qRT-PCR was performed in an ABI Prism 7500 Real-time system (Applied Biosystems), using Power SYBR green PCR master mix (Applied Biosystems). Primers at 400 nM (Table 6) and 50 ng of cDNA were used in each reaction. A first denaturation step of 10 min at 95°C was followed by amplification and quantification with 40 cycles of 15 s at 95°C and 1 min at 60°C. Primers
were determined according to the 2-
cDNA. Differences in the relative amounts of mRNA
iciency was analysed by RT-PCR using serial dilutions of
genome from Pseudomonas Genomedatebase (http://www.pseudomonas.com/); and their ef

Table 6. Primers used in this work.

| Gene   | Primer fw (5’-3’)                  | Primer rv (5’-3’)                  | Description               |
|--------|-----------------------------------|-----------------------------------|---------------------------|
| gltP   | GCAGATCTTTGCACCGATCTCT            | TTGAACCGGTCGGACGAAGCAC          | qRT-PCR                   |
| murA   | CATTCTCGGCGCCGAGAACT             | ATGCTGCTGGCGCCCTGGCTGGAG       | qRT-PCR                   |
| agmK/murU | AGGTGAGCTCTGGTGAGTCTG          | ACGCTCGGAGGTGAGCTGGAG          | qRT-PCR                   |
| nvaK   | AGGGCGGCGGTCTGACG                | ATTCGTCTTGGCTGACG              | qRT-PCR                   |
| mupP   | GCCGATCTTGACCGGCTG               | AGGGCGGCGGTCTGACG              | qRT-PCR                   |
| fosA   | ACCAGGGCGGTCTGACG               | AGGGCGGCGGTCTGACG              | qRT-PCR                   |
| rplU   | GCAAGGCGGCTGTGACGAGA            | CGGTGTGCTCTGACGAG             | Check DNA contamination in RNA samples |
| rpsL   | GCCGGCGGTCTGACGAGA            | CGGTGTGCTCTGACGAG             | Check DNA contamination in RNA samples |

rplU_Fw and rplU_Rv were used to quantify the expres-
sion of the housekeeping gene rplU (Table 6). Primer3
Input software was used to design the primers; their speciﬁcity was tested by BLAST alignment against P. aeruginosa genome from Pseudomonas Genome Data-
base (http://www.pseudomonas.com/); and their efﬁ-
ciency was analysed by RT-PCR using serial dilutions of
cDNA. Differences in the relative amounts of mRNA
were determined according to the 2-

Construction of fosA overexpressing mutants

The gene fosA was ampliﬁed using OE_fosA_Fw and
OE_fosA_Rv primers (Table 6). The oligonucleotides
contain the restriction enzyme target sequences of AvrI and BamHl, respectively, to allow the cloning of the ampiclon into the plasmid pSEVA234 (Table 1), previously digested using these restriction enzymes. The PCR product was puriﬁed from an agarose gel, by using a DNA puriﬁcation kit (GE Healthcare, Chicago, IL, USA) and, afterwards, cloned into the pGEM-T Easy vector, following supplier’s instructions. E. coli DH5α competent
cells were then transformed with these plasmids, which were afterwards puriﬁed using the QIAprep Spin mini-
prep kit 250 (Qiagen) and digested with AvrI and BamHl. The resulting fragments were puriﬁed from an agarose gel, and the plasmid pPLM001 (pSEVA234 fosA)(Table 1) was obtained through a ligation reaction by the
T4 DNA ligase (New England BioLabs, Ipswich, MA,
USA), transformation of E. coli DH5α competent cells
and puriﬁcation using the QIAprep Spin miniprep kit 250 (Qiagen). The preparation of competent P. aeruginosa PA14, Tgc5d, Tob5d and Caz5d cells was performed as previously described (Irani and Rowe, 1997) and the plasmids pPLM001 (pSEVA234 fosA overexpressing plasmid) or pSEVA234 (control plasmid) were then introduced by transformation. The resulting strains were

Measurement of intracellular fosfomycin

The wild-type strain PA14, and the resistant mutants
Tgc5d, Tob5d and Caz5d were grown in 20 ml of LB medium until exponential phase (OD600nm = 0.6). The cultures were centrifuged and each of the pellets sus-
pended in 1 ml of LB. Cells were incubated with 2 mg ml⁻¹ of fosfomycin for 60 min at 37°C and then washed three times with 1 ml of a buffer (10 mM Tris pH
7.3, 0.5 mM MgCl₂ and 150 mM NaCl) by centrifugation
at 7000 rpm during 10 min. In the case of the mutants carrying either pPLM001 or pSEVA234, cells were grown
during 90 minutes more with 1 mM of isopropyl-
β-D-1-thiogalactopyranoside (IPTG) after reaching the exponential phase, before the incubation with fosfomycin.
After the last wash, pellets were suspended in 60 µl of
0.85% NaCl. A 10 µl aliquot of each culture was serially
diluted and plated on LB agar to estimate number of colony-forming units (CFUs) after the incubation with fosfomycin. Cells from the remaining 50 µl suspension were broken at 100°C for 5 min and centrifuged at
13 200 rpm for 10 min. Then, paper disks (9 mm,
Macherey-Nagel, Düren, Germany) were soaked with
40 µl of each supernatant and plated on LB agar plates
previously seeded with Escherichia coli OmniMAX™
(Invitrogen, Waltham, MA, USA). The concentration of fosfomycin was quantified by measuring the diameter of the inhibition halos and by extrapolating these values to those from a standard curve of fosfomycin, obtained by measuring the hallo diameter produced by disks containing
known concentrations of fosfomycin, adjusted to a

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logarithmic equation. Intracellular fosfomycin concentration was normalized to the number of CFUs of each strain after the incubation with the antibiotic and is presented as the amount (μg) of fosfomycin per 10^9 cells.

As a control of cell death after the 100°C treatment, 10 μl of each culture was plated on LB agar plates and growth checked after incubation during 20 h at 37°C. No growth was detected in any case. As a control of fosfomycin stability after the 100°C treatment, 40 μl of fosfomycin at 50 mg ml⁻¹, treated or not at 100°C during 5 min, was soaked on disks and deposited on LB agar plates previously seeded with Escherichia coli OmniMAX™ and incubated at 37°C for 20 h.

**Growth curves measurement**

The growth of P. aeruginosa was analysed by measuring the absorbance OD₆₀₀nm of bacterial cultures. 10 μl of overnight bacterial cultures was added to 140 μl of LB in 96-well microtiter plates (Nunc™, Rochester, NY, USA), at a final OD₆₀₀nm of 0.01. Measures were made every 10 min during 20 h, in a Tecan Infinite 200 plate reader (Tecan, Männedorf, Switzerland) at 37°C. Six technical replicates were used to estimate the average value of absorbance for each strain.

**Determination of fosfomycin resistance mutant frequency**

The fosfomycin resistance mutant frequency was determined by plating 10^8 cells of each of the studied strains in LB plates containing different fosfomycin concentrations, from 32 to 256 μg ml⁻¹. Control plates without antibiotic were also seeded with sequential dilutions of the cultures. After 24 h at 37°C, the colonies were counted in order to determine the mutant frequency of each resistant mutant at each concentration and calculated as the ratio between colonies in presence and absence of fosfomycin. The values were determined as the average of three independent replicates for each strain and condition.

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**Conflict of interest**

The authors declare that they have no conflict of interest.

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Convergent collateral fosfomycin sensitivity

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