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Quantitative contribution of efflux to multi-drug resistance of clinical *Escherichia coli* and *Pseudomonas aeruginosa* strains

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ABSTRACT

Background: Efflux pumps mediate antimicrobial resistance in several WHO critical priority bacterial pathogens. However, most available data come from laboratory strains. The quantitative relevance of efflux in more relevant clinical isolates remains largely unknown.
Methods: We developed a versatile method for genetic engineering in multi-drug resistant (MDR) bacteria, and used this method to delete *tolC* and specific antibiotic-resistance genes in 18 representative MDR clinical *E. coli* isolates. We determined efflux activity and minimal inhibitory concentrations for a diverse set of clinically relevant antibiotics in these mutants. We also deleted *oprM* in MDR *P. aeruginosa* strains and determined the impact on antibiotic susceptibility.
Findings: tolC deletion abolished detectable efflux activity in 15 out of 18 tested *E. coli* strains, and modulated antibiotic susceptibility in many strains. However, all mutant strains retained MDR status, primarily because of other, antibiotic-specific resistance genes. Deletion of *oprM* altered antibiotic susceptibility in a fraction of clinical *P. aeruginosa* isolates.
Interpretation: Efflux modulates antibiotic resistance in clinical MDR isolates of *E. coli* and *P. aeruginosa*. However, when other antimicrobial-resistance mechanisms are present, inhibition of MDR efflux pumps alone is often not sufficient to restore full susceptibility even for antibiotics with a dramatic impact of efflux in laboratory strains. We propose that development of novel antibiotics should include target validation in clinical MDR isolates.
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1. Introduction

Multi-drug resistant (MDR) Gram-negative bacterial pathogens represent a major global threat to human health. MDR pathogens drive clinical usage of last-resort antibiotics such as carbapenems and colistin further amplifying resistance development and the emergence of pan-resistant pathogens [1]. As a consequence, deaths attributable to antimicrobial resistance may rise sharply, although the global burden of MDR remains difficult to estimate [2]. Resistance is multi-factorial but one promiscuous mechanism covering diverse antibiotic classes is the expression of so-called resistance-nodulation-division (RND) superfamily exporters, which mediate active efflux of small molecules including many antibiotics from the periplasm and the inner membrane to the extracellular environment [3–6]. RND efflux systems are tripartite complexes of an inner membrane pump that is driven by the proton-motive force, a periplasmic adapter protein and an outer membrane channel [3–6]. *Escherichia coli* has multiple RND efflux systems with different pumps and adaptor proteins, but all depend on a single outer membrane efflux protein, TolC [7]. *Pseudomonas aeruginosa* has 18 different RND efflux systems, and the major outer membrane efflux protein OprM is required for resistance to a wide variety of antibiotics under standard conditions, although overexpressed OpmJ or OmpH can replace OprM [7]. In laboratory strains of various Gram-negative bacterial pathogens, upregulation of RND efflux systems increases resistance to

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Research in context

Evidence before this study

We searched Pubmed and Google Scholar using the search terms 'efflux' and 'antibiotics' and 'resistance' for articles published up to September 1st, 2018. Multiple studies have shown that genetic inactivation of efflux in laboratory strains of *E. coli* and *P. aeruginosa* dramatically increases their susceptibility to approved antibiotics that are active exclusively against Gram-positive bacteria. If this key role of efflux also applies to more relevant multi-drug resistant (MDR) clinical isolates remains uncertain, since available genetic methods are cumbersome for such isolates. Alternative methods such as efflux inhibitors yield inconclusive results because of their pleiotropic effects and limited activity.

Added value of this study

This study seeks to quantify the impact of efflux in multi-drug resistance of WHO critical priority 1 pathogens. It demonstrates the utility of a versatile genetic method for generating mutants in multi-drug resistant clinical isolates, and reveals an only moderate contribution of efflux to antimicrobial resistance in clinical isolates of *E. coli* and *P. aeruginosa*.

Implications of all the available evidence

Whilst results from laboratory strains suggest efflux as a potentially useful target for novel antimicrobials, this study shows that inhibiting efflux might have limited impact on clinically relevant multi-drug resistant strains of *E. coli* and *P. aeruginosa*. These data demonstrate the importance of target validation in clinical isolates in addition to analysis of laboratory strains.

diverse antibiotics, while genetic inactivation of such systems renders mutants hypersensitive [3–6]. Overexpression of RND efflux systems is observed in many MDR clinical isolates suggesting that efflux might be involved in increasing resistance [8].

Importantly, several key antibiotics including macrolides, various tetracyclines, and fusidic acid are clinical effective against Gram-positive pathogens, but fail against Gram-negative bacteria primarily because of efflux [5,9]. Based on these observations, academia and industry have devoted major efforts to develop efflux inhibitors, hoping that such compounds could break the intrinsic resistance of Gram-negative bacteria against these already approved drugs [9]. However, almost all evidence for the impact of efflux comes from laboratory strains. In contrast to these strains, clinical MDR isolates evolve from diverse genetic backgrounds, acquire specific antibiotic-resistance determinants, upregulate various efflux pumps, and diminish outer membrane permeability. All these changes, as well as additional poorly characterized physiological differences, can influence the impact of efflux [10–12].

The quantitative contribution of efflux in clinical MDR isolates remains still largely unclear [3–6,13]. Common methods for genetically inactivating efflux are often cumbersome for MDR clinical strains [13]. Previous studies used such methods to determine the impact of a single RND efflux pump (AcrB-AcrA-TolC) in various *Escherichia coli* isolates, and a single or multiple RND efflux pumps in few *Pseudomonas aeruginosa* isolates [13–18]. Efflux inhibitors are widely used to assess efflux contributions in clinical strains, and often show only moderate impact on resistance [18–22]. However, available inhibitors block efflux in a substrate-dependent manner and inhibition might be incomplete when used at low concentrations, especially in clinical MDR strains, thus underestimating the role of efflux [5,6]. At high concentrations, these inhibitors have pleiotropic effects on cell envelope integrity and overall bacterial physiology, impairing conclusive interpretation [5]. Efflux gene expression and sequences can be readily determined, but overexpression and sequence polymorphisms of these genes poorly correlate with resistance levels in clinical isolates [23,24].

Here, we developed a method to generate genetically defined efflux mutants in diverse MDR isolates, as part of the Innovative Medicines Initiative (IMI) Translocation project [25]. We applied the method to diverse MDR clinical isolates of two major pathogens on the WHO priority list of particularly serious threats [1,26], *Escherichia coli* [27] and *Pseudomonas aeruginosa* [28]. We deleted genes encoding crucial outer membrane subunits of multiple RND pumps for extensive disruption of efflux, and determined the impact on efflux activity and antibiotic susceptibility.

2. Methods

2.1. Whole-genome sequencing and analysis

Escherichia coli clinical isolates were grown on plates overnight and colonies were re-suspended in PBS. DNA was extracted after lysozyme digestion at 37 °C for 15 min using a Maxwell 16 DNA extraction device (Promega, Mannheim, Germany). Bacteria were treated with lysis buffer containing Proteinase K and RNase for 1 h at 65 °C and DNA purification was performed as described by the manufacturer. After quality control of the DNA, a fragmentation library was generated as described by the manufacturer (NexteraXT kit, Illumina, San Diego, CA, USA). The genomes were sequenced as multiplexed samples using a 2 × 300 bp V3 reaction kit on an Illumina MiSeq instrument using 300 bp paired end mode to obtain an average coverage of approximately 70-fold for all isolates. After quality control, reads were quality trimmed and downstream analysis was carried out using CLC Genomics Workbench (Qiagen) and SeqSphere+ (Ridom). Reads were mapped to the reference *Escherichia coli* ATCC 25922 genome (GenBank: CP009072.1 (ATCC 25922) and 1855 genes belonging to the common core genome were analyzed for their allelic differences (core genome Multilocus Sequence Typing, cgMLST [29,30]). Multi-locus sequence types (MLST) were determined using the web server available at https://cge.cbs.dtu.dk//services/MLST/ using typing scheme ("MLST configuration") *Escherichia coli*#1 [31]. Acquired antibiotic resistance genes were identified using the database ResFinder [32]. Chromosomal point mutations associated with antibiotic resistance were identified usidng PointFinder [33]. To close plasmid sequences, we sequenced isolates EC03 and EC11 also with MinION using the 1D barcoded library preparation kit (EXP-NBD103, Oxford Nanopore Technologies) according to the manufacturer's protocol.

2.2. Gene deletion in clinical isolates

The choice of positive selection markers for the first recombination is severely limited for multi-drug resistant isolates, in which most standard antibiotic resistance cassettes confer no additional selectable phenotype. However, almost all our isolates were sensitive to the potassium salt of the tellurium oxyanion tellurite (TeO_3^{2-}) at concentrations of 50 mg l^{-1} (*E. coli*) or 200 mg l^{-1} (*Pseudomonas aeruginosa*). This enabled us to use a thiopurine-*S*-methyltranferase (*tpm*, ACIAD2922) from *Acinetobacter baylyi* as a positive selection marker that reduces tellurite to intracellular metallic tellurium (detectable as black colony colour), and might also convert tellurite into volatile dimethyl telluride [34]. In *E. coli*, *tpm* expression from the *rpsL* promoter of *Burkholderia cenocepacia* was sufficient to yield tellurite resistance. To obtain high-level resistance in *P. aeruginosa*, we expressed *tpm* from the strong constitutive *pX2* promoter [35]. It was important to use exponentially growing *Pseudomonas* for selection, as initially non-growing cells could eventually form colonies on tellurite plates even without the resistance cassette. Often we got both small and large colonies, the latter of which were more likely to carry the *tpm* cassette. We purified transconjugants by restreaking. For negative selection, we used classical

sucrose sensitivity conferred by levansucrase SacB from *Bacillus subtilis* in NaCl-free media [36].

We used *E. coli* JKE201 expressing the protein π (the initiator protein for R6K) for propagating and conjugation of the R6K-containing plasmids. Like its parental strain MFD*pir* [37], JKE201 is free from bacteriophage Mu. This phage is present in commonly used *E. coli* donor strains such as SM10λ*pir* posing a risk of phage contamination of exconjugants [37]. JKE201 (like MFD*pir*) also carries a *dapA* deletion conferring auxotrophy to diaminopimelic acid (DAP), which permits facile counter-selection in absence of DAP to obtain donor-free exconjugants. As additional features, apramycin/gentamicin resistance cassette and all three type IV restriction endonucleases as well as the type I restriction/modification EcoKI have been removed in JKE201 to improve cloning of PCR-amplified DNA fragments (genotype: MG1655 RP4-2-Tc::[ΔMu1::*Δaac(3)IV*::*lacI*^{q}-*ΔaphA-Δnic35*-ΔMu2::*zeo*] Δ*dapA*::*(erm-pir)* Δ*recA* Δ*mcrA* Δ(*mrr-hsdRMS-mcrBC*)) [38].

Plasmids carrying a fusion of 700 bp flanking regions of the gene of interest as well as a tellurite resistance cassette and *sacB* and the π-dependent R6K origin of replication (which cannot propagate in *P. aeruginosa* and *E. coli*), were constructed using Gibson assembly [39] and transferred into JKE201 by heat shock. Transformants were selected on LB agar plates containing 10 mg l^{-1} potassium tellurite and 100 μM 2,6-diaminopimelic acid. Plasmids were sequenced, and confirmed transformants were mated with clinical *E. coli* or *P. aeruginosa* strains on filters with 0·45 μm pores. Ex-conjugants were incubated for at least one hour in fresh LB before selection on LB plates containing 50 mg l^{-1} (*E.coli*) or 200 mg l^{-1} (*P. aeruginosa*) potassium tellurite. Ex-conjugants were validated by PCR and correct clones were grown in liquid LB to exponential phase and selected on agar plates containing NaCl-free LB and 20% (*E. coli*) or 10% (*P. aeruginosa*) sucrose. Deletion mutants were validated by PCR and confirmed by sequencing.

In several cases, this procedure rapidly yielded the desired deletion mutants. For many other strains, however, there was a strong bias for both single cross-overs to occur at the same flanking region thus restoring the wild-type locus instead of the desired deletion. Which flanking region was preferred differed from isolate to isolate and was apparently unrelated to the endogenous sequence of these flanking regions as determined by whole genome sequencing. In many cases, we could solve this problem by PCR-screening for rare clones that had used the non-preferred site for the first single cross-over. Such clones often resolved using the preferred region yielding the desired gene deletion mutant.

Plasmids of two *E. coli* strains were cured by inserting both *tpm* and *sacB* followed by extensive positive and negative selection rounds. One of the two strains, EC03, contained a plasmid with 99·95% sequence identity to previously characterized pH 105 [40]. Plasmid loss was verified by whole-genome sequencing.

2.3. Efflux assay

We determined efflux activities using a Nile Red assay as described [41]. In brief, *E. coli* overnight cultures in lysogeny broth (37 °C) were incubated with 10 μM carbonyl cyanide *m*-chlorophenylhydrazone and 5 μM Nile Red for 3 h at 37 °C and 1 h at room temperature. The cells were centrifuged, resuspended in buffer (1 mM $MgCl_2$, 20 mM potassium phosphate, pH 7·0), transferred to a 96-well plate, and placed in a plate reader (Synergy H4, BioTek). Fluorescence (excitation at 552 nm, emission 636 nm) was followed for 120 s. Nile Red efflux was then triggered by rapid energization with 50 mM glucose and monitored for another 300 s to detect even rather slow efflux.

3. Results

3.1. Gene deletion in clinical Escherichia coli isolates

We selected 24 *E. coli* clinical isolates from patient blood, sputum, surface swabs, urine, or fecal samples. All isolates were non-susceptible to agents of three or more antimicrobial categories thus fulfilling the standard definition for MDR status [42]. Whole genome sequencing revealed that these isolates were genetically diverse with hundreds of allelic differences, covered seven different multi-locus sequence types (STs) with the expected dominance of ST 131 [43] (Fig. 1a), and carried various resistance determinants (Fig. 1b). Taken together, these data demonstrate that our collection is representative of diverse, clinically relevant MDR *E. coli* strains.

We developed a method combining various previously described components, to facilitate efficient generation of genetically defined efflux mutants in these isolates. We employed a suicide plasmid (Fig. 1c) and two consecutive single cross-overs. We used thiopurine-*S*-methyltranferase Tpm conferring resistance to tellurite [44], as positive selection marker that works even in MDR isolates. Tellurite resistance has been previously used as positive marker [45–48]. We used levansucrase SacB conferring susceptibility to sucrose as negative selection marker. We accelerated plasmid construction using rapid Gibson assembly [39]. We transformed the plasmids into *E. coli* JKE201 [38] as

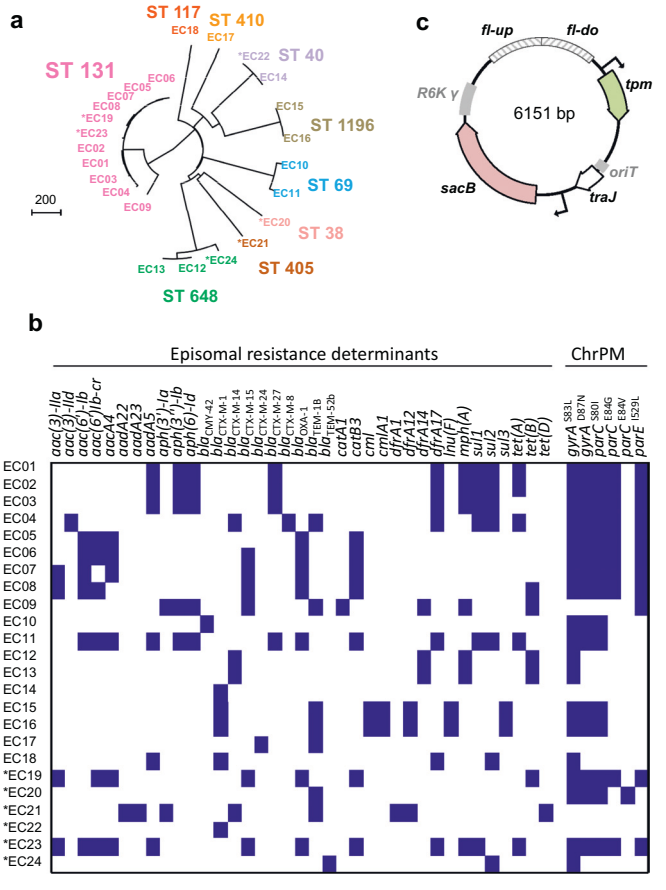

Fig. 1. Genome diversity of clinical *Escherichia coli* strains and geme deletion plasmid. (**a**,**b**) Analysis of clinical MDR *Escherichia coli* strains by whole genome sequencing. (**b**) Tree illustrating the relationship between 18 strains for which we could obtain Δ*tolC* mutants, based on the cgMLST (core genome multi-locus sequence typing) allelic profiles. The scale bar represents 200 allelic differences. The tree is colored according to MLST sequence types (STs). Strains EC19 to EC24, for which were unable to obtain Δ*tolC* mutants are marked with asterisks. (**c**) Occurrence of acquired antimicrobial resistance genes as detected by ResFinder [32] and chromosomal point mutations (ChrPM) associated with antimicrobial resistance as identified by PointFinder [33]. (**c**) Plasmid for deleting genes in MDR bacterial pathogens. The plasmid carries the *R6K γ* origin of replication which depends on the replication protein π (encoded by *pir*) which is absent in almost all clinical strains; *tpm* encoding thiopurine-*S*-methyltranferase, which confers resistance to tellurite (most MDR clinical isolates are sensitive to tellurite); the origin of conjugational transfer *oriT*; *traJ* encoding the transcriptional activator for conjugational transfer genes; *sacB* encoding levansucrase, which confers sensitivity to sucrose. The hatched regions *fl-up* and *fl-do* represent flanking regions of the target gene for deletion. The hooked arrows represent promoters.

a donor for conjugation. This strain that was free of commonly encountered phages that could infect clinical isolates and distort mutant phenotypes [37]. For efficient selection of ex-conjugants against donor cells, we exploited the fact that JKE201 lysed without supplementation of diaminopimelic acid. We realized that gene deletion often failed when there was a strong bias for recombination in one of the two flanking regions. As both the first and the second single cross-over occurred in the same preferred region, we mostly got reversion back to wild-type, instead of the desired deletion. To mitigate this problem, we determined in which flanking region the first single cross-over occurred using PCR with primers binding to upstream and downstream sequences as well as primers binding to plasmid sequences. We then selected rare ex-conjugants in which the first cross-over had occurred in the non-preferred region, and used them for subsequent selection for second cross-over. This yielded desired deletion mutants at increased rates.

To determine the contribution of efflux, we aimed at deleting *tolC*, which encodes the outer membrane channel required for the function of all known *E. coli* RND efflux systems [7]. For five strains, we could obtain clean deletions of *tolC* within three days, while for the others we obtained plasmid insertions that reverted to wild-type upon the second cross-over in all tested clones. We repeated the conjugations and got seven additional mutants. For additional rounds, we specifically worked with ex-conjugants with non-preferred insertions sites, which yielded six more mutants in three attempts. Together, we obtained *tolC* mutants for 18 out of 24 *E. coli* clinical isolates. For six isolates, we did not detect the desired deletion even after screening up to 296 different colonies from five independent attempts. However, sequence types and resistance gene patterns of these isolates were largely represented by the 18 successfully manipulated strains (Fig. 1a,b). We therefore did not put further efforts into generating mutants for the six failed isolates. All *tolC* deletions were confirmed by sequencing.

3.2. Efflux activities of E. coli ΔtolC mutants

To determine efflux activities of *E. coli* isolates and their respective *tolC* mutants, we used a Nile Red-based assay [41] that is especially suitable for comparing efflux in diverse isolates [49] (Fig. 2a,b; Supplementary Table 1). Seventeen *E. coli* isolates showed rapid efflux with kinetics in the range of previously reported data [41] (a representative examples is shown as black line in Fig. 2a). Deletion of *tolC* totally abolished efflux in 15 strains and dramatically slowed down efflux in two other strains (Fig. 2a,b). The molecular mechanism of residual slow glucose-dependent Nile Red fluorescence loss in these two *tolC* mutants remain unknown, but may involve outer membrane efflux proteins that could partially compensate for TolC [50], or other efflux mechanisms that do not depend on a particular outer membrane efflux protein. EC18 and EC18 Δ*tolC* showed declining fluorescence signals in energy-depleted cells even before re-energization with glucose, preventing quantitative analysis of energy-dependent efflux (dotted blue and orange lines in Fig. 2a). Together, our data indicate complete inactivation of efflux upon *tolC* deletion in 15 out of 18 clinical isolates.

3.3. Antibiotic susceptibility of E. coli ΔtolC mutants

We tested the strains for susceptibility to therapeutically relevant antimicrobials according to EUCAST (European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables. Version 9.0, 2019) using commercial phenotyping systems (Vitek 2 and *E*-tests, bioMérieux). We represent the data as minimal inhibitory concentrations that prevent growth (MIC) (Fig. 2c,d; Supplemental Table 1). High MIC values correspond to probable clinical treatment failures ("resistant").

We first tested three antibiotics with potent Gram-positive antibacterial activity, but poor activity against Gram-negative bacteria (doxycycline, erythromycin, and fusidic acid). Previous studies showed that in *E. coli* laboratory strains, intrinsic resistance against these compounds is mainly caused by RND efflux pumps [5,9]. We confirmed these findings for a Δ*tolC* mutant of the laboratory strain *E. coli* K-12 MG1655 that showed eightfold, tenfold, and more than 64fold lower MIC values compared to parenteral MG1655 (first arrows in each panel of Fig. 2c). Δ*tolC* mutants of several MDR *E. coli* clinical isolates also became more susceptible (Fig. 2c). For these three drugs, susceptibility breakpoints for *E. coli* and PK/PD breakpoints are not available. Based on EUCAST breakpoints for Gram-positive bacterial pathogens as tentative first approximations, several Δ*tolC* mutants might indeed have become sensitive to clinically achievable doxycycline or erythromycin concentrations (hatched blue area). Breakpoints might also differ somewhat for efflux-inhibited *E. coli* as compared to Gram-positive bacteria. Surprisingly, however, several other mutants retained high resistance well above putative breakpoints. As most of these mutants showed no detectable efflux activity (Supplemental Table 1), they might possess other resistance mechanisms.

Deletion of *tolC* in MDR *E. coli* isolates had some impact on MIC values of antibiotics that are active against *Enterobacteriaceae* (Fig. 2d). The effect size was generally lower or similar to what has been observed for fully susceptible laboratory strains [3–6], and almost always too small to convert resistant strains into susceptible ones (i.e., a shift from red to blue areas). We had expected larger effects since many clinical strains show increased expression of RND pumps, and decreased outer membrane permeability, which can further enhance the impact of efflux [10–12]. Several strains showed very high MIC values beyond the detection range of the phenotyping systems. In these cases, deletion of *tolC* might have made the strains more susceptible without being noticed. However, such changes would be still far from clinically relevant concentrations. For β-lactam antibiotics (alone or in combination with β-lactamase inhibitors), changes occurred sometimes in the opposite direction (i.e., getting more resistant) as previously reported in an efflux inhibitor study [51]. This could be a consequence of pleiotropic effects of efflux inactivation.

The only exceptions with substantial loss of resistance at least against some antibiotics were isolates EC08, EC10, EC14, and EC17. EC10 became susceptible to the aminoglycosides tobramycin and amikacin, and trimethoprim-sulfamethoxazole, upon *tolC* deletion (thick blue arrows in Fig. 2d). Interestingly, EC10 was the only isolate that showed resistance to these drugs but lacked antibiotic-specific resistance determinants (such as *aac(6′)-Ib* and *aac(6′)-Ib-cr* encoding aminoglycoside modifying enzymes, of *dfrA* alleles and *sul* alleles resistant to trimethoprim or sulfamethoxazole, respectively; Fig. 1b). This apparent lack of specific resistance mechanisms would be compatible with an important role of general mechanisms such as efflux, although aminoglycosides have not yet been found to be relevant substrates for RND efflux systems in *E. coli*. Trimethoprim might directly interact with RND pumps [52]. On the other hand, EC10 Δ*tolC* remained resistant to agents in three antimicrobial categories and thus retained MDR status. Another mutant, EC08 Δ*tolC*, showed diminished MIC for amikacin (MIC 4 mg l^{-1}) but retained tobramycin resistance. Under these circumstances, the amikacin MIC may not be a reliable predictor of clinical activity [53] and the strain should be reported as "intermediate" for amikacin according to EUCAST rules [54] (dotted blue arrow in Fig. 2d). Strains EC14 and EC17 became susceptible to ciprofloxacin upon *tolC* deletion. Common chromosomal *gyrA* target mutations conferring fluoroquinolone resistance were absent in these two isolates (Fig. 1c).

3.4. Role of efflux-independent mechanisms

The modest impact of efflux inactivation in many clinical isolates was initially surprising, but did not necessarily indicate a minor role of efflux. Inactivating TolC can cause pleiotropic side effects including suppression of outer membrane porin F (OmpF) [7,51,55,56]. Such pleiotropic effects might provide compensatory resistance mechanisms not

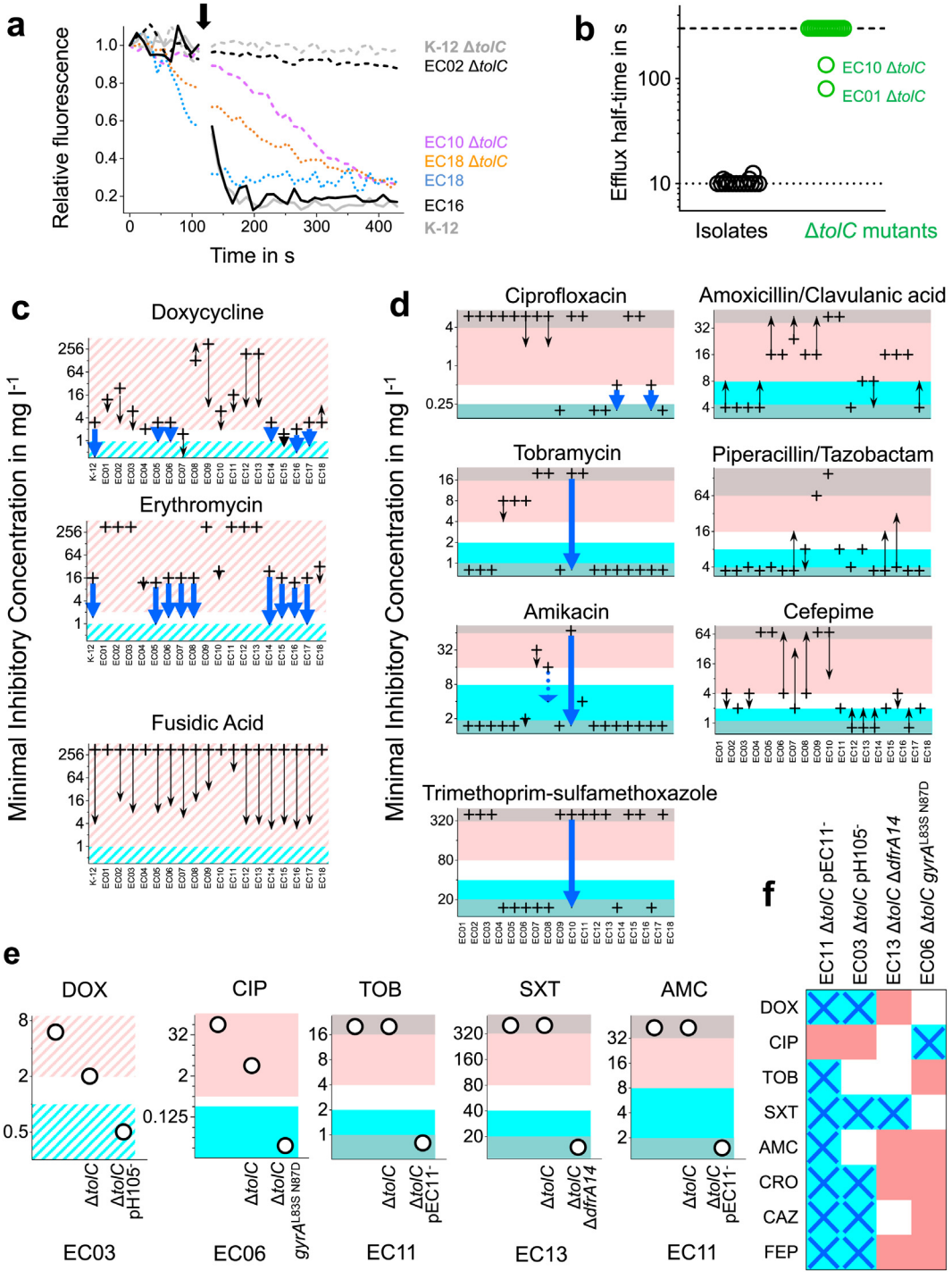

Fig. 2. Impact of genetic inactivation of efflux on antimicrobial susceptibility in MDR *E.coli*. (**a**,**b**) Efflux activities in clinical isolates and corresponding Δ*tolC* mutants. (**a**) Energy-depleted cells were loaded with Nile red. Cells were then re-energized with glucose (arrow, 120 s), and efflux was measured as decrease in Nile red fluorescence (which is lower in aqueous solution compared to bacterial membranes). All isolates except EC18 showed rapid energy-dependent efflux (representative example shown in black), whereas all Δ*tolC* mutants showed no, or much slower, fluorescence loss upon energization (examples shown as dashed black or magenta lines). EC18 (blue dotted line) and its Δ*tolC* mutant (orange dotted line) lost fluorescence in an energy-independent manner (i.e., even prior to glucose addition) preventing quantitative analysis of efflux. Representative traces for the laboratory strain K-12 and its Δ*tolC* mutant are shown for comparison (grey lines). (**b**) Time intervals after energization until 50% of fluorescence intensity was lost. Time resolution prevented measurement of half-times below 10 s (dotted line) or above 300 s (dashed line). (**c**,**d**) Minimal inhibitory concentrations that prevent growth (MICs) of *Escherichia coli* isolates and corresponding Δ*tolC* mutants. Data are shown for drugs that are normally ineffective against *Escherichia coli* (**c**), and for common therapeutically used antimicrobials (**d**). Crosses represent values for parental isolates. The impact of Δ*tolC* deletion is represented by arrows. If there is no arrow, the mutant MIC remained at the parental level. MIC ranges corresponding to clinical resistance (red) or susceptibility (blue) according to EUCAST breakpoints are shown as background. Breakpoints shown in (c) are estimates based on values for other bacterial pathogens. MIC values outside the measurement range are shown as shaded areas. The thick blue arrows mark conversion of clinical resistance to susceptibility as a result of genetic inactivation of major efflux systems, while the dotted blue line for EC08 and amikacin should still be reported as "intermediate" (see text). K-12 had MIC values below the lowest measured concentration (shaded blue regions) for all antibiotics shown in (d). (**e**,**f**) Impact of additional inactivation of specific resistance determinants in efflux-deficient strains. (**e**) Comparison of susceptibility of parental strains, their Δ*tolC* mutants, and various double mutants (AMC, Amoxicillin/Clavulanic acid; SXT, Trimethoprim-sulfamethoxazole). We determined susceptibility to ciprofloxacin using a broth microdilution technique to cover the nanomolar concentration range. (**f**) Antimicrobial spectrum changes in double mutants (blue, switch from "resistant" or "intermediate" in the *tolC* mutant to "susceptible" in the double mutant; white, "susceptible" unaltered; red, "resistant" unaltered; CAZ, Ceftazidime; CIP, Ciprofloxacin; CRO, Ceftriaxone; DOX, Doxycycline; FEP, Cefepime; TOB, Tobramycin; TZP, Piperacillin/Tazobactam). The blue crosses indicate resistance mechanisms that were specifically inactivated in each of the four double mutants.

present in the wild-type isolates such as restricting drug entry through OmpF [57], thus replacing efflux with little overall change in MIC. This was suggested as a possible explanation for increased resistance to β-lactams upon efflux inhibition in *Salmonella* [51]. Alternatively, the non-impressive Δ*tolC* phenotypes could be due to the large sets of antibiotic-modifying enzymes, acquired antibiotic-resistant target alleles such as *dfrA14*, and/or mutated chromosomal target genes such as *gyrA*$^{S83L\ D87N}$ (Fig. 1b). To determine the quantitative contributions of efflux/compensatory side effects vs. efflux-independent antibiotic-specific mechanisms, we constructed double mutants in isolates from three different *E. coli* sequence types.

To inactivate multiple resistance mechanisms simultaneously, we cured plasmids carrying multiple antibiotic-modifying genes and acquired antibiotic-resistant target alleles (Fig. 3) in two strains yielding double mutants EC11 Δ*tolC* pEC11^{-} and EC03 Δ*tolC* pH 105^{-}. We constructed two additional double mutants in which a single resistance determinant was targeted: EC13 Δ*tolC* Δ*dfrA14* with potentially restored susceptibility to trimethoprim; and EC06 Δ*tolC gyrA*$^{L83S\ N87D}$ with potentially restored susceptibility to ciprofloxacin. This combined inactivation of efflux and antibiotic-specific mechanisms led to full susceptibility to one or several of the six tested clinically relevant antimicrobial categories in strict concordance with the respective targeted mechanisms (Fig. 2e,f). These data indicated that the *tolC* deletion, which was present in each double mutant, did not have compensatory side effects that would confer clinically relevant resistance to the tested antibiotics. By contrast, antibiotic-specific mechanisms were crucial. Together, this evidence suggested that inhibiting RND efflux alone was largely insufficient to break MDR of *E. coli* clinical isolates, even in case of antibiotics with Gram-positive activity such as doxycycline that were thought to lack Gram-negative activity primarily because of efflux.

3.5. Antibiotic susceptibility of *P. aeruginosa* ΔoprM mutants

We also selected several MDR *P. aeruginosa* isolates from four different Belgian hospitals [58] that had divergent serotypes, antibiograms and efflux pump expression patterns (Fig. 4a–d; Supplemental Table 2). In seven out of 12 tested *P. aeruginosa* strains, we were able to delete *oprM* encoding the outer membrane channel of major efflux systems MexAB and MexXY (except for rare isolates of the taxonomic outlier PA7 group [59], in which MexXY uses OprA), as well as minor systems MexMN, MexVW, and partially MexJK. For some extensively MDR *P. aeruginosa* isolates, there was a high background growth even at 200 mg l^{-1} tellurite hampering positive selection for ex-conjugants. All mutants were verified by sequencing.

We first tested two antibiotics that are active against other bacteria, but fail against *P. aeruginosa* (tigecycline and azithromycin). In *P. aeruginosa* laboratory strains, inactivation of a single OprM-dependent efflux pump, MexAB breaks this intrinsic resistance [5], suggesting that efflux inhibition could extend the spectrum of these already approved antibiotics to *P. aeruginosa*. In several of our clinical MDR isolates, *oprM* deletion indeed increased sensitivity to both antibiotics (Fig. 4b). Based on EUCAST breakpoints for *Enterobacteriaceae* as tentative first approximation, several Δ*oprM* mutants became almost susceptible (hatched blue area). Some strains also showed substantial sensitization to azithromycin. Since MIC values in standard Müller-Hinton broth might underestimate clinical efficacy [60], efflux inhibition could be sufficient for therapeutic potency also for this antibiotic and these particular strains. Breakpoints might differ somewhat for efflux-inhibited *P. aeruginosa* compared to *Enterobacteriaceae* and Gram-positive bacteria. However, unexpectedly, several of Δ*oprM* mutants of our clinical strains retained high resistance well above putative breakpoints for both antibiotics even after inactivation of efflux.

In several strains, deletion of *oprM* had remarkable effects for aztreonam (which is rarely used against this pathogen because of often high MIC values [61]) and a ceftazidime / avibactam combination that was approved in 2015, after isolation of the *P. aeruginosa* strains used in this study [58] (Fig. 4c). The latter data supported a previously suggested partial contribution of efflux in pre-existing resistance against this new drug combination [62].

MDR *P. aeruginosa* Δ*oprM* mutants showed mostly non-impressive alterations in susceptibility to antibiotics with anti-pseudomonal activity. These data extend observations from a previous study, in which six strains from an industrial collection showed moderate impact of *oprM* inactivation on high resistance to anti-pseudomonal antibiotics [18]. Strain PA256 Δ*oprM* had a diminished MIC value for amikacin (6 mg l^{-1}) but remained resistant to tobramycin. Under these

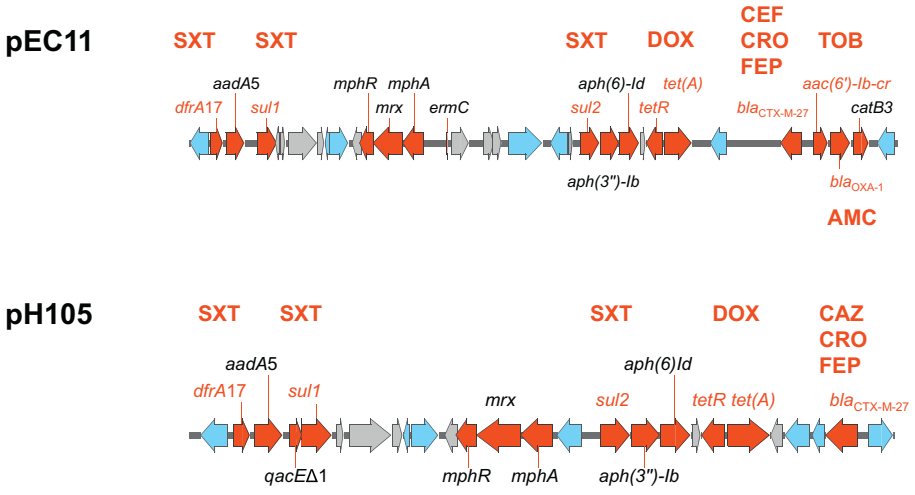

Fig. 3. Plasmid-encoded antimicrobial resistance genes. Gene clusters that contained all detected antimicrobial resistance genes on plasmids present in EC11 (pEC11; 30,000 bp of a total plasmid sequence of 148,945 bp are shown) and EC03 (pH 105; 20,000 bp of a total plasmid length of 134,920 bp). Gene names in red indicate genes conferring resistance to clinically relevant antibiotics that were tested in this study (AMC, Amoxicillin/Clavulanic acid; CAZ, Ceftazidime; CRO, Ceftriaxone; DOX, Doxycycline; FEP, Cefepime; SXT, Trimethoprim-sulfamethoxazole SXT; TOB, Tobramycin). AAC(6′)-Ib-cr can modify both tobramycin and amikacin. However, strain EC11 was only resistant to tobramycin while amikacin MIC remained below the EUCAST breakpoint (Fig. 2d). Similar findings have been obtained for many *Enterobacteriaceae* that possess AAC(6′)-Ib [53]. AAC(6′)-Ib-cr can also cause low-level resistance to ciprofloxacin [65], but in EC11 which carries also a high-resistance chromosomal *gyrA*$^{S83L\ D87N}$ allele (Fig. 1c), AAC(6′)-Ib-cr likely provided only a modest contribution to overall ciprofloxacin resistance. Gene names in black indicate other resistance genes. Transposable elements are shown in blue, and other genes are grey.

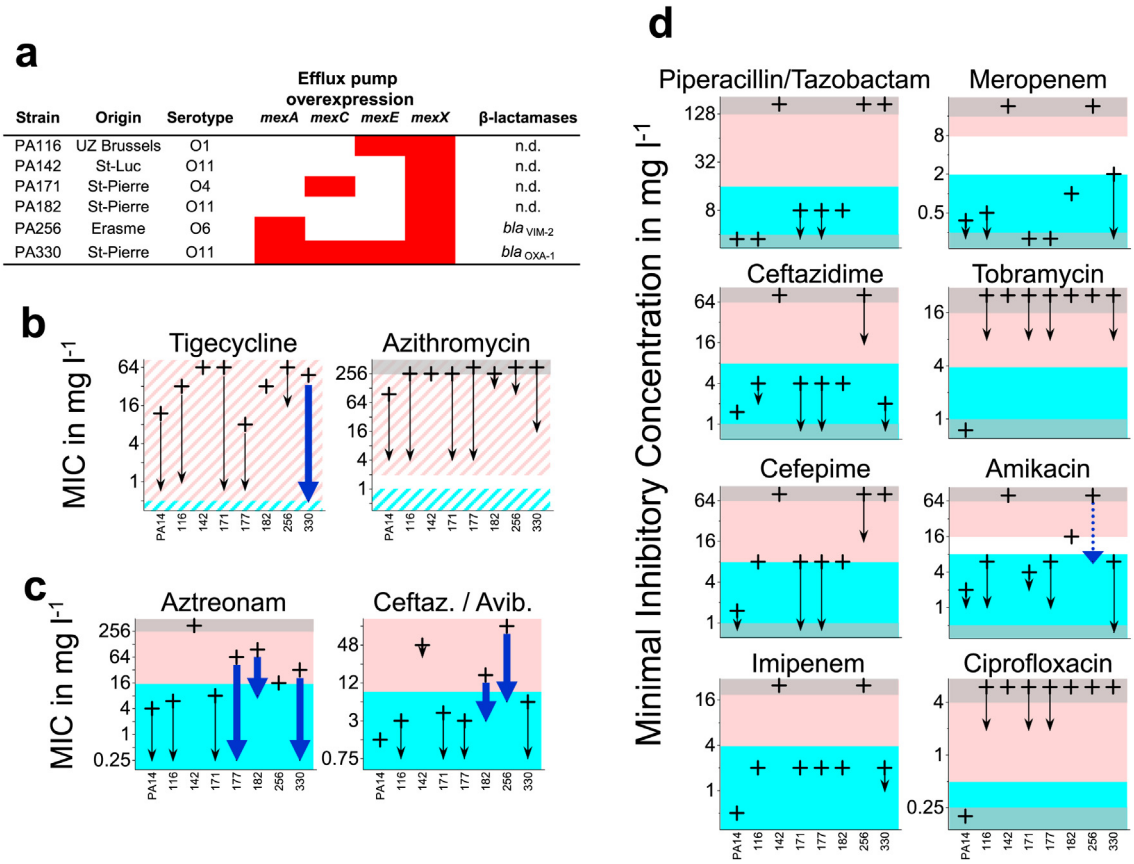

Fig. 4. Impact of *oprM* deletion on MDR *P. aeruginosa* clinical isolates. (**a**) Strain characteristics of highly resistant *Pseudomonas aeruginosa* clinical isolates (n.d., not detected). (**b**,**c**,**d**) Minimal inhibitory concentrations that prevent growth (MICs) of clinical *P. aeruginosa* strains and their Δ*oprM* mutants. The laboratory strain UCBPP-PA14 (PA14) and its Δ*oprM* mutant are shown for comparison. Data represent drugs that are ineffective against *P. aeruginosa* (**a**), that are rarely used or only recently approved (**b**) (Ceft. / Avib., Ceftazidime / Avibactam), or commonly used against *P. aeruginosa* (**c**). Crosses represent values for parental isolates. The impact of *oprM* deletion is represented by arrows. If there is no arrow, the mutant MIC remained at the parental level. MIC ranges corresponding to clinical resistance (red) or susceptibility (blue) according to EUCAST breakpoints are shown as background. MIC values outside the measurement range are shown as shaded areas. Breakpoints shown in (**b**) are estimates based on values for other bacterial pathogens. The thick blue arrows mark conversion of clinical resistance to susceptibility as a result of genetic inactivation of major efflux systems, while the dotted blue line for PA256 and amikacin should still be reported as "resistant" (see text). MIC values outside the measurement range are shown above the highest tick, or below the lowest tick, respectively.

circumstances, the amikacin MIC may not be a reliable predictor of clinical activity, and the strain should be reported as "resistant" for amikacin (dotted blue arrow in Fig. 4d).

Deletion of *oprM* inactivates the clinically most relevant RND efflux systems in *P. aeruginosa*, MexAB and MexXY, which represent the primary targets for efflux inhibitor development programs against MDR *P. aeruginosa*. In addition, *P. aeruginosa* encodes various other RND efflux systems that are independent of OprM [5]. It is possible that such other efflux systems masked the effect of *oprM* deletion in some of our mutants (as it has been shown for one single isolate of the rare PA7 group [17]), but the most important alternative pumps, MexCD-OprJ or MexEF-OprN, were not overexpressed in our most refractory strains 142 and 256 (Fig. 4a). It might be challenging to develop promiscuous but safe inhibitors/inhibitor combinations for all relevant *P. aeruginosa* efflux systems, given the toxicity of several otherwise promising inhibitors [3–6,9].

4. Discussion

Our genetically defined mutants show that major RND efflux systems contribute to intrinsic resistance against antibiotics with Gram-positive activity, and to the MDR phenotype of many MDR clinical *E. coli* and *P. aeruginosa* strains. However, the effects were only moderate in most isolates, and did not generally increase susceptibility to clinically relevant levels. In several cases, additional mutagenesis revealed a dominant impact of antibiotic-specific resistance determinants, which maintained high resistance levels even in absence of efflux. These data are compatible with previous genetic studies for single RND efflux pump (AcrB-AcrA-TolC) in various *Escherichia coli* isolates, or individual or multiple RND efflux pumps in some *Pseudomonas aeruginosa* isolates [13–18]. Studies employing efflux inhibitors also suggested often only moderate impact on resistance in clinical isolates [18–22].

A number of our efflux mutants became susceptible to antibiotics with exclusive Gram-positive activity as expected from data for laboratory strains. However, surprisingly, some mutants remained non-susceptible suggesting additional resistance mechanisms. *E. coli* or *P. aeruginosa* might carry such mechanisms in spite of being already intrinsically resistant, for two potential reasons. First, even sub-lethal exposure to antibiotics can provide sufficient selection for evolution of high-level resistance [63]. Second, large integrons carrying multiple resistance determinants can be exchanged between Gram-positive and Gram-negative bacteria, and this can include resistance determinants such as *tetAR*, that might have adaptive roles predominantly in Gram-positive bacteria. Laboratory strains, which most previous studies analyzed, had no recent opportunities for gene-exchange with Gram-positive bacteria and no previous exposure to these antibiotics, resulting in discrepancies in the role of efflux when compared to clinical MDR isolates.

Taken together, our data do not generally support previous hopes for broadening the spectrum of approved antibiotics to Gram-negative critical priority bacterial pathogens using efflux inhibitors, or for restoring activity of other antibiotics [3,4]. These results thus argue against the

main motivation for developing such inhibitors. On the other hand, some strains did show substantial sensitization upon efflux inactivation. If such strains could be rapidly identified in the clinics, efflux inhibitors could find some applications.

Our study has limitations and caveats. In particular, we investigated several key classes of antibiotics in the two major pathogens that have been at the focus of RND efflux research, but there might be some other drug-bug combinations, for which efflux inhibitors could still be useful, especially in case of entirely new compound classes. It is also important to note that some residual efflux activity might be mediated by alternative outer membrane channels, especially in *P. aeruginosa* Δ*oprM* mutants. Some residual efflux might also still be present in *E. coli* Δ*tolC* mutants, although the Nile Red efflux assay showed non-detectable activities in most of our mutants. Inactivating the major RND outer membrane channel is thus not necessarily the same as inhibiting efflux completely. Efflux might also play an important role in strains with only low-level resistance, which we did not study: However, this would have limited relevance for solving the urgent MDR crisis.

Future studies could determine the role of efflux in clinical multidrug resistance in additional pathogens using the methods developed in this study. Further research on RND efflux systems and their substrate selectivity is essential to obtain a rational basis for developing efficacious novel drugs that escape efflux. Efflux systems also contributes to pathogen virulence [64] suggesting a potential role for efflux inhibitors in anti-virulence strategies. Some efflux inhibitors have also antimicrobial properties [6], and could thus be valuable as starting points for novel antibiotics.

In summary, this study shows a moderate contribution of efflux to antimicrobial resistance in MDR clinical strains of the WHO top priority pathogens *E. coli* and *P. aeruginosa*. More generally, we demonstrate the importance of validating potential target genes in relevant clinical isolates, in addition to studying well-characterized laboratory strains. Our gene deletion method will be suitable for this purpose.

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Conflict of interest statement

CK and VT have a pending patent application. We have no other conflicts to declare.

Author contributions

D.B. designed the study with input from A.E. and C.K.; O.C. and J.M.B. constructed mutants; D.M., O.C., H.S.-S., and R.N. determined genome sequences; O.C. and P.M. determined efflux activities; A.E. and J.F. determined MIC values; P.S.A., V.T., C.K., J.K., and C.D. provided tools and expertise; D.B. wrote the manuscript with early input from O.C. and subsequently all authors provided advice and approved the final manuscript.

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