1 Interchangeability of Periplasmic Adaptor Proteins AcrA and 2 AcrE in forming functional efflux pumps with AcrD in *Salmonella* 3 Typhimurium

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15 Running title: AcrD can function with AcrA or AcrE

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29 Abstract

30 Background

31 RND efflux pumps are important mediators of antibiotic resistance. RND pumps
32 including the principal multidrug-efflux pump AcrAB-TolC in *Salmonella*, are tripartite
33 systems, with an inner membrane RND-transporter, a periplasmic adaptor protein
34 (PAP) and an outer membrane factor (OMF). We previously identified the residues
35 required for binding between the PAP AcrA and the RND-transporter AcrB and have
36 demonstrated that PAPs can function with non-cognate transporters. AcrE and
37 AcrD/AcrF are homologues of AcrA and AcrB, respectively. Here, we show that AcrE
38 can interact with AcrD, which does not possess its own PAP, and establish that the
39 residues previously identified in AcrB-binding are also involved in AcrD-binding.

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41 Methods

42 The *acrD* and *acrE* genes were expressed into a strain lacking *acrABDEF* (Δ 3RND).
43 PAP residues involved in promiscuous interactions were predicted based on
44 previously defined PAP-RND interactions and corresponding mutations generated in
45 *acrA* and *acrE*. Antimicrobial susceptibility of the mutant strains was determined.

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47 Results

48 Co-expression of *acrD* and *acrE* significantly decreased susceptibility of the Δ 3RND
49 strain to AcrD substrates showing that AcrE can form a functional complex with
50 AcrD. The substrate profile of *Salmonella* AcrD differed from that of *E. coli* AcrD.
51 Mutations targeting the previously defined PAP-RND interaction sites in AcrA/AcrE
52 impaired efflux of AcrD-dependent substrates.

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54 **Conclusions**

55 These data indicate that AcrE forms an efflux-competent pump with AcrD and thus
56 presents an alternative PAP for this pump. Mutagenesis of the conserved
57 RND-binding sites validates the interchangeability of AcrA and AcrE, highlighting
58 them as potential drug targets for efflux inhibition.

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79 Introduction

80 Multidrug-resistance (MDR) efflux pumps play a major role in antibiotic resistance of
81 bacteria by reducing the intracellular concentration of drugs^{1, 2}. In particular, the
82 Resistance-Nodulation-Division (RND) family of efflux pumps contribute to clinically
83 relevant antibiotic resistance in Gram-negative bacteria, such as *Salmonella*
84 *enterica*³⁻⁶. Tripartite RND pumps span the double membrane of Gram-negative
85 bacteria and consist of an inner membrane RND-transporter, a periplasmic adaptor
86 protein (PAP) and an outer membrane factor (OMF)⁷⁻⁹. Owing to their unique
87 organisation, tripartite efflux pumps can directly export drugs across the outer
88 membrane to the extracellular environment. The majority of RND pumps exhibit a
89 broad substrate profile, which includes multiple classes of antibiotics, biocides,
90 detergents, dyes and metals^{10, 11}. Furthermore, there is increasing evidence that
91 RND pumps also play a role in various biological processes and behaviours,
92 including biofilm formation, detoxification, quorum sensing and virulence¹²⁻¹⁴.

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94 In *S. enterica*, five RND pumps have been characterised: AcrAB, AcrD, AcrEF,
95 MdtABC and MdsABC⁵. The AcrAB pump is constitutively expressed in *S. enterica*
96 and displays a remarkably wide substrate profile, consisting of multiple classes of
97 antibiotics, bile salts, detergents and dyes⁵. The AcrEF system possesses a similar
98 substrate profile to AcrAB but is not constitutively expressed^{5, 15}. In *S. enterica*, AcrB
99 is 80% identical to AcrF, whereas AcrD is 64% and 65% identical to AcrB and AcrF,
100 respectively^{16, 17}. This sequence divergence is reflected in the substrate profile of
101 AcrD, which exhibits markedly narrower substrate range compared to AcrB and
102 AcrF. In *Escherichia coli*, AcrD has been shown to export aminoglycosides and
103 anionic β -lactams¹⁸⁻²⁰. Compared to AcrB, AcrD has a stronger preference for

anionic β -lactams, which is linked to differences in the access binding pocket²¹. Homology modelling of *E. coli* AcrD combined with molecular dynamic simulations have also suggested that the different substrate specificities between AcrB and AcrD stem from the corresponding differences in the physicochemical and topological properties of their binding pockets²². Until now, this view of AcrD substrate selectivity has been assumed to also apply to the AcrD pump in *S. enterica*.

In *Salmonella*, most of the RND pumps require the OMF TolC to form a functional tripartite complex, the exception being MdsABC, which can function with either MdsC or TolC²³. The RND-transporter genes are usually co-located with their cognate PAP on a single operon. In *S. enterica* there are four RND-associated PAPs: AcrA, AcrE, MdtA and MdsA⁵. Based on sequence analyses and structural alignments, AcrA and AcrE have been shown to be the most closely related, with a sequence identity of 69.3% over their first 374 residues as calculated by Expasy SIM server²⁴, and just a single gap in the alignment of this region, which maps to the signal sequence and does not impact the mature protein. Correspondingly, AcrA and AcrE share a predicted secondary structure (Fig. 1a)²⁵, which is also nearly identical to that of the experimentally determined structure of the AcrA from *E. coli*, with the exception of the divergent C-terminal region, which is predicted to be disordered and is not seen in the available cryo-EM structures^{8,9}. This allows creation of reliable homology models of AcrE, which we have previously reported²⁵. In contrast to AcrA and AcrE, both MdtA and MdsA are more sequentially divergent, with MdsA sharing less than 30% identity with AcrA and AcrE, which is predicted to also translate in differences into significant differences 3D structure⁵.

the homology model of the AcrE²⁵. The non-conserved substitutions are shown in sidechain and semi-transparent sphere representation. The mapping demonstrates that the bulk of the discrepancies, which may be expected to account for the functional differences between the PAPs map to their beta-barrel and membrane-proximal domains.

Although AcrA is the cognate PAP for AcrB, the RND pump AcrD was shown to depend on AcrA to form a functional tripartite efflux system since it lacks is not encoded with its own PAP²⁶. Indeed, AcrA has been reported to also function with AcrF in *E. coli*²⁷ and recently, AcrE has been demonstrated to function with AcrB in *S. Typhimurium*²⁵.

The major RND-transporter binding residues of AcrA have been highlighted by cryogenic electron microscopy structural studies^{8, 9} and validated by mutagenesis²⁵. Our comparative analysis of *Salmonella* PAPs demonstrated that these critical residues fall within a discrete number of linear sequence sites, which we termed RND-binding boxes²⁵. These are shared between AcrA and AcrE, potentially explaining their interchangeability²⁵. However, MdtA and MdsA are not interchangeable and cannot function with non-cognate RND-transporters and correspondingly are significantly different within RND-binding boxes²⁵. Although AcrA and AcrE have been shown to be largely interchangeable, the ability of AcrE to function with AcrD remains unknown.

Here, we have investigated the substrate specificity of *S. Typhimurium* SL1344 AcrD. We furthermore explored whether the interoperability of AcrA and AcrE

extends to the RND-transporter AcrD and whether this interaction is driven by the same residues that have been shown to be important for other PAP-RND combinations.

Materials and methods

Bacterial strains

All strains used in this study are listed in Table 1. The *Salmonella enterica* serovar Typhimurium strains were derived from the wild-type strain SL1344 (henceforth referred to as *S. Typhimurium*), a pathogenic strain first isolated from an experimentally infected calf²⁸. All strains were grown in Luria–Bertani (LB) broth at 37°C with aeration.

Construction of gene deletion mutants

The Δ *acrB* mutant strain was constructed previously²⁹. All other mutant strains were constructed using the λ red recombinase system described previously, antibiotic markers were removed, and the process repeated to make double and triple knockout *S. Typhimurium* SL1344 strains (Table 1)³⁰. All the primers used for generating gene knockouts and cloning are listed in Table S1.

Plasmid construction

All plasmids used in this study are listed in Table 1. The *acrD* and *acrA* genes were amplified from *S. Typhimurium* SL1344 by PCR and cloned into pHSG398 and pACYC177 plasmids, respectively, as described previously²⁶. Expression of the *acrE* gene is repressed by H-NS¹⁵. Therefore, to clone *acrE* into pACYC177 and obtain sufficient expression, a forward primer was designed containing the *trc* promoter and

the *acrE* ribosomal binding site (Table S1). The synthetic *trc* promoter is derived from the *E. coli trp* and *lacUV5* promoters that drives high level of transcription³¹.

The *acrE* gene was amplified from *S. Typhimurium* SL1344 genomic DNA by PCR using the *acrE* cloning F and R primers (Table S1), which introduced *Scal* and *Bam*HI sites, respectively. The PCR fragment contained the *trc* promoter and a region 14 base pairs upstream to 2 base pairs downstream of *acrE*. This fragment was digested with *Scal* and *Bam*HI and cloned into the corresponding sites of pACYC177, where an ampicillin resistance gene was located. The resulting plasmid pACYC177 *acrE* solely possessed a kanamycin resistance marker. The control pACYC177Kan^R plasmid was constructed as described previously²⁶.

Construction of mutant *pacrA* and *pacrE* plasmids

The *acrA* and *acrE* point mutants were generated using the GeneArt[®] Gene Synthesis Service (Invitrogen, Germany) and subsequently cloned into the pACYC177 plasmid using the Subcloning Service (Invitrogen, Germany). All plasmids were sequenced to check for the presence of the desired point mutations and to ensure there were no unwanted secondary mutations.

Determination of antimicrobial susceptibility

The minimum inhibitory concentration (MIC) of various antimicrobials was determined using the agar dilution method according to CLSI guidance³².

229 **Table 1. List of *S. enterica* serovar Typhimurium strains and plasmids used in**
230 **this study.**

Strain/ plasmid	Genotype/characteristic	Source/reference
Strains		
SE01	Wild-type <i>S. Typhimurium</i> SL1344	28
SE02	$\Delta acrB$	29
SE446	$\Delta acrB$ /pHSG398	This study
SE449	$\Delta acrB$ / <i>pacrD</i>	This study
SE379	$\Delta acrD$	This study
SE502	$\Delta acrAB\Delta acrD\Delta acrEF$ ($\Delta 3RND$)	This study
SE544	$\Delta 3RND$ /pHSG398	This study
SE545	$\Delta 3RND$ /pACYC177Kan ^R	This study
SE507	$\Delta 3RND$ / <i>pacrD</i>	This study
SE510	$\Delta 3RND$ / <i>pacrA</i>	This study
SE511	$\Delta 3RND$ / <i>pacrE</i>	This study
SE506	$\Delta 3RND$ /pACYC177Kan ^R + pHSG398	This study
SE548	$\Delta 3RND$ /pACYC177Kan ^R + <i>pacrD</i>	This study
SE508	$\Delta 3RND$ / <i>pacrD</i> + <i>pacrA</i>	This study
SE509	$\Delta 3RND$ / <i>pacrD</i> + <i>pacrE</i>	This study
SE546	$\Delta 3RND$ /pHSG398 + <i>pacrA</i>	This study
SE547	$\Delta 3RND$ /pHSG398 + <i>pacrE</i>	This study
SE553	$\Delta 3RND$ /pHSG398 + <i>pacrA</i> F292G	This study
SE554	$\Delta 3RND$ /pHSG398 + <i>pacrA</i> G363F	This study
SE554	$\Delta 3RND$ /pHSG398 + <i>pacrE</i> F291G	This study
SE556	$\Delta 3RND$ /pHSG398 + <i>pacrE</i> G362F	This study
SE557	$\Delta 3RND$ / <i>pacrD</i> + <i>pacrA</i> F292G	This study
SE558	$\Delta 3RND$ / <i>pacrD</i> + <i>pacrA</i> G363F	This study
SE559	$\Delta 3RND$ / <i>pacrD</i> + <i>pacrE</i> F291G	This study
SE560	$\Delta 3RND$ / <i>pacrD</i> + <i>pacrE</i> G362F	This study
Plasmids		
pACYC177	vector; Amp ^R , Kan ^R	ATCC
pACYC177Kan ^R	vector; Kan ^R	This study
pHSG398	vector; Chl ^R	Takara Bio Group
<i>pacrA</i>	SL1344 <i>acrA</i> gene cloned into pACYC177; Kan ^R	This study
<i>pacrE</i>	SL1344 <i>acrE</i> gene cloned into pACYC177; Kan ^R	This study
<i>pacrD</i>	SL1344 <i>acrD</i> gene cloned into pHSG398; Chl ^R	This study
<i>pacrA</i> F292G	SL1344 <i>acrA</i> gene with a F292G point mutation cloned into pACYC177; Kan ^R	This study
<i>pacrA</i> G363F	SL1344 <i>acrA</i> gene with a G363F point mutation cloned	This study

	into pACYC177; Kan ^R	
<i>pacrE</i> F291G	SL1344 <i>acrE</i> gene with a F291G point mutation cloned into pACYC177; Kan ^R	This study
<i>pacrE</i> G362F	SL1344 <i>acrE</i> gene with a G362F point mutation cloned into pACYC177; Kan ^R	This study

Amp^R, ampicillin resistant; Chl^R, chloramphenicol resistant; Kan^R, kanamycin resistant

Results and discussion

AcrD of *S. Typhimurium* SL1344 does not transport aminoglycosides

Despite being isolated several decades ago³³, the substrate specificity of AcrD remains relatively poorly characterised experimentally. Therefore, an additional rationale of this study was to investigate the substrate specificity of *S. Typhimurium* SL1344 AcrD, especially in the context of PAP-RND interactions, which may provide modulatory effects on the specificity of the pump. Previously, it has been reported that *E. coli* AcrD exports aminoglycosides^{19, 20}. However, there is a lack of experimental evidence in *Salmonella* and most of the features of *Salmonella* AcrD are inferred based on close sequence similarity to *E. coli* AcrD (97.4%, Fig. S1). While, some previous work has addressed this, aminoglycosides have not been specifically investigated^{5, 17}. Therefore, we investigated the substrate range of AcrD in *S. Typhimurium* SL1344.

The Δ *acrD* SL1344 strain did not exhibit any significant increase in susceptibility to any of the antimicrobials tested as previously reported¹⁷. This is likely because expression of *acrD* is generally low in laboratory conditions and for many compounds, any effect would be masked by the presence of AcrB¹⁷. Therefore, *pacrD* was transformed into the Δ *acrB* strain, and the effect of *acrD* overexpression

on antimicrobial susceptibility of the resulting transformant was determined. The $\Delta acrB/pacrD$ strain displayed significantly increased MIC values to reported AcrD-substrates aztreonam, carbenicillin, cloxacillin, fusidic acid, nafcillin, novobiocin and oxacillin (Table 2) consistent with previous studies²⁶, suggesting that protein is functionally expressed and incorporated into the membrane. Surprisingly, the introduction of *acrD* into $\Delta acrB$ ($\Delta acrB/pacrD$) strain did not result in a significant increase in MICs to the aminoglycosides kanamycin, gentamicin, spectinomycin or streptomycin (Table 2), implying that AcrD is not measurably contributing to aminoglycoside efflux. This is in contrast to the reported role of AcrD in the aminoglycoside resistance of *E. coli*, wherein deletion of *acrD* was shown to decrease aminoglycoside MICs by two to eight fold¹⁹ and expression of *acrD* from a plasmid in an *acrB::aph* $\Delta acrD$ strain increased aminoglycoside MICs by two fold³⁴. In agreement with our findings, the AcrD efflux pump of the Gram-negative plant pathogen *Erwinia amylovora* has also been reported to not play a role in aminoglycoside resistance³⁵. Similarly, our data shows that the AcrD efflux pump of *S. Typhimurium* SL1344 does not seem to be involved in aminoglycoside export as previously assumed.

A possible explanation for the differences in the substrate profiles of AcrD between *E. coli* and *S. Typhimurium* could be the observed discrepancy between the residues in their respective access and deep binding pockets (Fig. S1). Due to the lack of experimental AcrD structure, the functional significance of the residues of the respective drug binding pockets of AcrD is inferred from their positional homology with corresponding AcrB residues, structures of which have been experimentally defined for both *E. coli*³⁶⁻³⁸, and more recently for *Salmonella*³⁹. Specifically, the

presence of a serine in the deep binding pocket of *S. Typhimurium* AcrD at position 610, which in *E. coli* AcrD is occupied by an alanine, could possibly impact the previously described lipophilic character of the drug binding cavity²². There are also two additional discrepancies which could be seen as non-conservative substitutions, namely that of *E. coli* AcrD isoleucine to a phenylalanine at position 633 (I633F) in *S. Typhimurium*, and leucine to a glutamine at position 565 (L565Q), both of which are likely to cause steric hinderance and impact the electrostatics of the access binding pocket, respectively^{21, 22}. These subtle differences may account for the notable differences in substrate recognition by AcrD between the two species.

AcrE forms a functional PAP-RND pair with AcrD

AcrD has been previously shown to depend on AcrA to function as an efflux system²⁶. Therefore, owing to the high similarity of the predicted RND-binding sites between the PAPs AcrA and AcrE^{25, 40}, we hypothesised that AcrE should also function with AcrD. To test this, we deleted the *acrAB*, *acrD* and *acrEF* genes in *S. Typhimurium* SL1344 to give a strain without active RND-dependent efflux, as indicated by significantly increased susceptibility to AcrB-, AcrF-, and AcrD-substrates (Table 2 and S2). The MdtABC and MdsABC systems are much less similar to the three AcrB/AcrD/AcrF-based systems and play a minor role in resistance. Consistent with this, they are not expressed under standard laboratory conditions⁵ and furthermore their inactivation did not have any additive effect on antimicrobial susceptibility^{5, 25}. Hence, these systems were not inactivated.

Firstly, we validated the previously reported AcrA dependency of AcrD in *S. Typhimurium* SL1344²⁶. The *pacrA* and *pacrD* plasmids were co-transformed into the

$\Delta 3RND$ strain, and the antimicrobial susceptibility of the resulting transformant was determined. We found that co-expression of *acrA* and *acrD* in the $\Delta 3RND$ strain significantly decreased susceptibility to known AcrD-substrates aztreonam, carbenicillin, cloxacillin, fusidic acid, nafcillin, novobiocin, oxacillin and ticarcillin (Table 2).

Secondly, to determine whether AcrE and AcrD form a functional complex together, *pacrD* and *pacrE* were co-transformed into the $\Delta 3RND$ strain and the susceptibility to validated AcrD-substrates was tested. Co-expression of *acrE* and *acrD* in the $\Delta 3RND$ strain significantly increased the MICs of aztreonam, carbenicillin, cloxacillin, fusidic acid, nafcillin, novobiocin, oxacillin and ticarcillin (Table 2). There was no difference in MIC values between co-expressing *acrD* with *acrA* or *acrE*, which demonstrates the full interchangeability of the two PAPs (Table 2). Furthermore, co-expression of either *acrE* and *acrD* or *acrA* and *acrD* in the $\Delta 3RND$ strain did not increase MIC values to the tested AcrB-substrates (i.e., acriflavine, crystal violet, ethidium bromide, erythromycin, methylene blue, rhodamine 6G and tetracycline)⁵, clearly showing AcrD-mediated efflux (Table S2). Overexpression of either *acrD* or *acrE* alone in the $\Delta 3RND$ strain did not significantly increased MIC values to the AcrD-substrates tested (Table 2), signifying that AcrE requires the presence of AcrD to form a functional, efflux-competent complex.

Our data suggests interchangeability between AcrA and AcrE in *S. Typhimurium* SL1344. One possible explanation for the interoperability between AcrA and AcrE is that the latter may function as a backup PAP for when AcrA function is impaired or lost. This idea is supported by evidence from studies that demonstrated that in *S.*

Typhimurium, in the absence of *acrA*, it was possible to select for *acrE* overexpression^{25 41}. Another study demonstrated that in the absence of *acrA* and *acrE*, it is possible to restore the phenotypic defect in active efflux by complementing with either *acrA* or *acrE*⁴⁰.

Disruption of the RND-binding residues in AcrA or AcrE impairs AcrD-mediated efflux of substrate drugs

Previously, we showed that the promiscuity between *Salmonella* AcrA and AcrE in their ability to form a functional complex with AcrB stems from the highly conserved RND-binding sites (termed RND-binding boxes) between these two PAPs. Specifically, within the *Salmonella* AcrA, we identified several residues mapping to the β -barrel and membrane proximal domains that were important for AcrB-binding²⁵. There, the disruption of the F292 or G363 residues in AcrA produced the most pronounced phenotypic effect, resulting in severely abrogated active efflux and significantly increased susceptibility to AcrB-substrates²⁵. Therefore, to investigate whether these residues are also important for binding of the newly determined cognate PAPs to AcrD, the point mutation corresponding to F292G or G363F were constructed in both *pacrA* and *pacrE* (F291G and G362F) respectively and co-transformed with *pacrD* into the $\Delta 3\text{RND}$ strain. Based on structural analysis, we chose F292G and G363F as target mutations due to their radical change of respective site-chain properties.

Consistent with the data obtained in coexpression with AcrB²⁵, the disruption of F292 or G363 in AcrA resulted in impaired AcrD-mediated efflux of AcrD-substrates, confirming that the same residues required for binding of AcrA to AcrB are also

required for its binding to AcrD (Table 2). These point mutations do not impact the protein levels and folding as previously demonstrated²⁵. To determine whether the corresponding residues in AcrE are also important for AcrD-binding, F291 and G362 were mutated (Fig. S2). As expected, the F291G or G362F point mutations in AcrE also impaired AcrD-mediated efflux in the $\Delta 3\text{RND}$ strain (Table 2). These data suggest that the PAP-RND binding sites previously identified based upon AcrA-AcrB interaction, are indeed both sequentially and functionally conserved between AcrA and AcrE and account for the productive recognition and formation of functional tripartite pumps.

Concluding remarks

Here, we report that the PAP AcrE can form a functional complex with the RND-transporter AcrD, further validating the interchangeability between the homologous PAPs AcrA and AcrE. Furthermore, this interchangeability is likely to be due to the highly conserved and specific RND-binding sites between these two PAPs. Our report highlights the redundancy between these two PAPs must be taken into account when targeting them for efflux inhibition. Therefore, the residues we identified here could inform future design of effective efflux inhibitors targeting PAPs or tripartite complex assemblies.

Table 2. Susceptibility of *S. Typhimurium* strains to antimicrobials.

Strain	MIC (mg/L)											
	ATM	CAR	CXA	FA	NAF	NOV	OXA	TIC	GEN	SPR	STR	KAN
Wild type SL1344	0.06	4	512	1024	1024	512	512	4	0.5	16	8	1
Δ <i>acrB</i>	0.06	1	4	4	8	2	4	1	0.25	16	8	1
Δ <i>acrB</i> /pHSG398	0.06	1	4	4	8	2	2	1	0.25	16	4	1
Δ <i>acrB</i> / <i>pacrD</i>	0.25	8	16	64	64	8	16	16	0.25	16	4	1
Δ <i>acrD</i>	0.06	4	512	1024	1024	512	512	4	0.5	16	4	1
Δ <i>acrAB</i> Δ <i>acrD</i> Δ <i>acrEF</i> (Δ 3RND)	0.06	1	1	4	2	1	1	1	0.25	16	4	1
Δ 3RND/pHSG398	0.06	0.5	1	4	2	1	1	1	0.25	16	4	1
Δ 3RND/pACYC177Kan ^R	0.06	1	1	4	2	1	1	1	0.5	16	4	>32
Δ 3RND/ <i>pacrD</i>	0.06	0.5	1	4	2	1	1	1	0.25	16	4	1
Δ 3RND/ <i>pacrA</i>	0.06	1	1	4	2	1	1	1	0.5	16	4	>32
Δ 3RND/ <i>pacrE</i>	0.06	1	1	4	2	1	1	1	0.5	16	4	>32
Δ 3RND/pACYC177Kan ^R + pHSG398	0.06	0.5	1	4	2	1	1	1	0.25	16	8	>32
Δ 3RND/pACYC177Kan ^R + <i>pacrD</i>	0.06	1	1	4	2	1	1	1	0.25	16	4	>32
Δ 3RND/ <i>pacrD</i> + <i>pacrA</i>	0.25	8	16	128	32	8	16	8	0.5	16	4	>32
Δ 3RND/ <i>pacrD</i> + <i>pacrE</i>	0.25	8	16	128	32	8	16	8	0.5	16	4	>32
Δ 3RND/pHSG398 + <i>pacrA</i>	0.06	0.5	1	4	2	1	1	1	0.5	16	4	>32
Δ 3RND/pHSG398 + <i>pacrE</i>	0.06	0.5	1	4	2	1	1	1	0.5	16	4	>32
Δ 3RND/pHSG398 + F292G <i>acrA</i>	0.06	0.5	1	4	2	1	1	1	0.5	16	4	>32
Δ 3RND/pHSG398 + G363F <i>acrA</i>	0.06	0.5	1	4	2	1	1	1	0.5	16	4	>32
Δ 3RND/pHSG398 + F291G <i>acrE</i>	0.06	0.5	1	4	2	1	1	1	0.5	16	4	>32
Δ 3RND/pHSG398 + G362F <i>acrE</i>	0.06	0.5	1	4	2	1	1	1	0.5	16	4	>32
Δ 3RND/ <i>pacrD</i> + F292G <i>acrA</i>	0.06	0.5	1	4	2	1	1	1	0.5	16	4	>32
Δ 3RND/ <i>pacrD</i> + G363F <i>acrA</i>	0.06	1	1	4	2	1	1	1	0.25	16	4	>32
Δ 3RND/ <i>pacrD</i> + F291G <i>acrE</i>	0.06	0.5	1	4	2	1	1	1	0.25	16	4	>32
Δ 3RND/ <i>pacrD</i> + G362F <i>acrE</i>	0.06	1	1	4	2	1	1	1	0.25	16	4	>32

ATM, aztreonam; CAR, carbenicillin; CXA, cloxacillin; FA, fusidic acid; GEN, gentamicin;

KAN, kanamycin; NAF, nafcillin; NOV, novobiocin; SPR, spectinomycin, STR, streptomycin, TIC,

ticarcillin

Values in bold indicate a significant increase (>2-fold) than those of their corresponding parental

strains.

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396 **Transparency declaration**

397 None to declare.

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