

Fosfomycin resistance in *Acinetobacter baumannii* is mediated by efflux through a major facilitator superfamily (MFS) transporter—AbaF

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Objectives: To decipher the function of A1S_1331, named AbaF (*Acinetobacter baumannii* Fosfomycin efflux), one of the primary targets of AbsR25, a small RNA of *A. baumannii*.

Methods: *abaF* was cloned in a multicopy plasmid and expressed from its native promoter in an efflux-deficient strain—*Escherichia coli* KAM32. Drug susceptibility, accumulation and efflux of ethidium bromide (EtBr) were determined in this strain. *abaF* was disrupted in *A. baumannii* using homologous recombination and its effect on drug susceptibility, biofilm formation and virulence was studied. Expression of *abaF* was followed by quantitative PCR in fosfomycin-challenged *A. baumannii* and fosfomycin-resistant mutants of *A. baumannii*. Expression of *abaF* in clinical strains of *A. baumannii* was determined by RT-PCR.

Results: Expression of *abaF* in *E. coli* KAM32 resulted in increased resistance to fosfomycin. Lower accumulation and higher efflux of EtBr from this strain confirmed the role of AbaF as an efflux pump. Disruption of *abaF* in *A. baumannii* caused an increase in fosfomycin susceptibility and a decrease in biofilm formation and virulence. The expression of *abaF* was higher in *A. baumannii* cells exposed to fosfomycin and in cells resistant to higher concentrations of fosfomycin. The clinically relevant strains of *A. baumannii* also tested positive for the expression of *abaF*.

Conclusions: The results of this study suggest that efflux is an important mechanism of fosfomycin resistance and AbaF is involved in fosfomycin resistance in *A. baumannii*. AbaF also seems to play a role in biofilm formation and virulence of *A. baumannii*.

Introduction

Acinetobacter baumannii is one of the leading causes of hospital-acquired infections worldwide. This Gram-negative coccobacillus is responsible for opportunistic infections of the skin, blood-stream, urinary tract and other soft tissues, and for ventilator-associated pneumonia.¹ The ability of the bacterium rapidly to develop resistance to antimicrobials and the isolation of MDR and pan-drug-resistant *A. baumannii* has focused worldwide attention on this bacterium.² The WHO has declared it as one of the most serious ESKAPE organisms.³ Multidrug resistance in *A. baumannii* is believed to be the result of enzymatic degradation of drugs, modification of the targets or active efflux of the drugs. The presence of chromosomally encoded cephalosporinases, the basal-level expression of chromosomally encoded drug efflux pumps and decreased membrane permeability corroborate this belief.^{4–6} As far as drug efflux is concerned, *A. baumannii* has been reported to harbour efflux proteins from all of the major efflux pump families, except the ABC transporter superfamily. These pumps efflux out a variety of unrelated drugs, such as aminoglycosides, quinolones, fluoroquinolones, macrolides,

tetracyclines, chloramphenicol, erythromycin and tigecycline.⁷ As the number of efflux pumps being characterized increases, adaptive drug resistance, i.e. overexpression or mutation of the intrinsic drug-resistance mechanisms according to the environment, is an important area of concern.⁸

Fosfomycin is an antibacterial agent that has been used for treatment of urinary tract infections as it retains activity in the relatively acidic conditions of the urinary tract.⁹ It inhibits the synthesis of the bacterial cell wall by targeting and inactivating UDP-N-acetyl-glucosamine-3-*o*-enolpyruvyltransferase.^{10,11} It was discovered as long ago as 1969 and is therefore not a new antibacterial agent. However, interest in fosfomycin is on the increase because of its antibacterial activity against drug-resistant, Gram-negative bacteria.^{12,13} Although *A. baumannii* is intrinsically resistant to fosfomycin monotherapy, there are some interesting reports on the use of fosfomycin in combination with other drugs, such as colistin, minocycline and polymyxin B.^{14,15} Despite the fact that *Acinetobacter* spp. are resistant to fosfomycin, there is a dearth of literature on the mechanisms responsible for this resistance. The common mechanisms of resistance to fosfomycin, such as modification of the glucose phosphate uptake proteins and

target proteins and enzymatic modification of fosfomycin itself, have not been described for *A. baumannii*.¹⁶ Also, there are so far no reports suggesting efflux-mediated fosfomycin resistance in any bacterial species.

In one of our earlier reports, we described a regulatory small RNA, AbsR25, in *A. baumannii*, which was implicated in the regulation of a putative efflux pump with the locus tag A1S_1331.¹⁷ Here we describe the biological function of A1S_1331, which is constitutively expressed in *A. baumannii* ATCC 17978. It seems to play an important role in fosfomycin resistance by pumping out the drug; hence we named it AbaF (*A. baumannii* Fosfomycin efflux). However, an equally important finding is the role of this efflux pump in biofilm formation and virulence of *A. baumannii*.

Materials and methods

Bacterial strains, plasmids and primers

All the bacterial strains, plasmids and primers used in this study are described in Tables S1 and S2 (available as Supplementary data at JAC Online).

Cloning and expression of *abaF* in *Escherichia coli* KAM32

abaF was cloned with its native promoter and terminator in pUC18, resulting in plasmid pUC18_abaF as described in the supplementary methods (available as Supplementary data at JAC Online). This plasmid was transformed into *E. coli* KAM32 cells resulting in *E. coli* KAM32/pUC18_abaF. The unmodified plasmid pUC18 was also transformed into *E. coli* KAM32 cells, resulting in *E. coli* KAM32/pUC18.

In vitro antimicrobial susceptibility assay

MICs of various antibiotics and ethidium bromide (EtBr) were determined by the broth microdilution method, using 96-well plates, in Mueller–Hinton medium (Merck, Germany), according to CLSI guidelines.¹⁸ Growth was monitored by OD readings at 600 nm (OD₆₀₀) after 12 h of incubation using a Spectramax plus plate reader (Molecular Devices, USA).

EtBr accumulation and efflux assay

EtBr fluorescence assays were performed as described earlier with suitable modifications.¹⁹ Cells from log phase cultures of *E. coli* KAM32/pUC18 and *E. coli* KAM32/pUC18_abaF were collected by centrifugation, washed and resuspended in PBS to OD₆₀₀ = 0.3.

For the EtBr accumulation assay, glucose and EtBr were added to the cell suspension, at a final concentration of 0.4% (w/v) and 10 mg/L, respectively. For EtBr efflux assay, EtBr, at a final concentration of 10 mg/L, was added to the cell suspension and incubated at 37°C for 15 min to facilitate loading of cells with EtBr. The efflux was initiated by adding glucose at a final concentration of 0.4% (w/v). The fluorescence of EtBr was measured over time using a Spectramax M2e plate reader (Molecular Devices, USA), in an opaque plate, at emission and excitation wavelengths of 610 and 480 nm, respectively.

The efflux pump inhibitor and energy decoupler CCCP was added in both the assays, where mentioned, to obtain a final concentration of 25 mg/L. Both the assays were performed thrice and relative fluorescence of EtBr was plotted against time elapsed.

Construction of disruption mutant of *abaF* and complementation

The detailed construction of the disruption mutant of *abaF* and its complementation is described in the supplementary methods. Briefly,

an internal region of *abaF* was cloned in an allele exchange vector, pMo130, resulting in plasmid pMo_RTabaF. This plasmid was mobilized to *A. baumannii* via conjugal transfer.²⁰ Transconjugant cells in which insertional inactivation of *abaF* had occurred due to recombination with the plasmid were designated as *A. baumannii* Δ *abaF*. Plasmid-borne expression of *abaF* was achieved in *A. baumannii* Δ *abaF*, resulting in the strain *A. baumannii* *pabaF*.

Fosfomycin treatment and total RNA isolation

Actively growing *A. baumannii* cells were treated with increasing concentrations of fosfomycin (0, 256, 512 and 1024 mg/L, corresponding to 0 \times , 1 \times , 2 \times and 4 \times MIC, respectively) for 2 h. After 2 h, the cells were harvested and total RNA was isolated from the cells using RNA-Xpress reagent (HiMedia, India) followed by DNase I treatment (Thermo Fisher Scientific, USA), according to the manufacturer's instructions. cDNA was synthesized from 1 μ g total RNA using random hexamer primer (Qiagen, USA) and SuperScript[®] III reverse transcriptase (Life Technologies, USA) as per the manufacturer's instructions.

Selection of fosfomycin-resistant mutants

Fosfomycin-resistant *A. baumannii* cells were selected by repetitive growth in the presence of increasing concentrations of fosfomycin (detailed in the supplementary methods). Total RNA from these fosfomycin-resistant cells was isolated and reverse transcribed to cDNA.

Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed as described earlier using LightCycler 480 SYBR Green I master mix and is outlined in the supplementary methods.¹⁷

Biofilm formation and *Caenorhabditis elegans* survival assay

The effect of disruption of *abaF* in *A. baumannii* on biofilm formation was studied and is described in the supplementary methods. WT *C. elegans* strain N2 was used for the survival assay performed as reported previously,²¹ and is detailed in the supplementary methods.

Results

AbaF decreases fosfomycin susceptibility of *E. coli* KAM32

E. coli KAM32 is a double knockout of efflux genes *ydhE* and *acrB*, making it deficient in efflux of antibiotics.²² Of the antibacterial compounds tested, a 16-fold decrease in susceptibility to fosfomycin was observed for *E. coli* KAM32/pUC_abaF as compared with *E. coli* KAM32/pUC18 (Table 1). The MIC values of kanamycin were 8-fold higher and those of chloramphenicol, minocycline, clindamycin, tetracycline, nalidixic acid and EtBr were 2-fold higher for *E. coli* KAM32/pUC_abaF. No significant change in the MIC values of other antibacterials was observed.

Fluorimetric assays using EtBr establish AbaF as an efflux pump

Since EtBr is a common substrate for most efflux pumps, fluorimetric assays using EtBr were performed to determine whether AbaF was involved in the active efflux mechanism. The accumulation of EtBr was lower in *E. coli* KAM32 cells expressing AbaF (Figure 1a) than in *E. coli* KAM32/pUC18. Addition of the energy

decoupler and efflux inhibitor CCCP caused an increase in accumulation of EtBr in both types of cells and the accumulation finally plateaued over time.

Similarly, when *E. coli* KAM32/pUC18_abaF and *E. coli* KAM32/pUC18 cells loaded with EtBr were energized by glucose, the efflux of EtBr (as measured by the decrease in fluorescence) was higher in the case of *E. coli* KAM32/pUC18_abaF (Figure 1b). The addition of CCCP caused the efflux to cease and the EtBr fluorescence started to build up in both types of cells owing to the inhibition of cellular efflux mechanisms.

Table 1. MICs of various compounds for *E. coli* KAM32/pUC18 and *E. coli* KAM32/pUC18_abaF

Compound	MIC (mg/L)	
	<i>E. coli</i> KAM32/pUC18	<i>E. coli</i> KAM32/pUC18_abaF
Fosfomycin	2	32
Chloramphenicol	0.25	0.50
EtBr	4	8
Minocycline	0.5	1
Tetracycline	0.06	0.12
Nalidixic acid	2	4
Kanamycin	4	32
Clindamycin	2	4
Gentamycin	1	1
Tobramycin	1	1
Streptomycin	4	4
Amikacin	0.5	0.5
Ofloxacin	0.01	0.01
Chlorhexidine	0.25	0.25
Ciprofloxacin	0.0015	0.0015
Trimethoprim	0.01	0.01
Erythromycin	0.5	0.5

Disruption of *abaF* increases susceptibility of *A. baumannii* to fosfomycin

Overexpression of *abaF* in *E. coli* KAM32 suggested the involvement of this transporter in efflux of fosfomycin and subsequently a role in resistance to the drug. To assess the importance of *AbaF* in *A. baumannii*, the *abaF* ORF was disrupted, which resulted in an 8-fold increase in susceptibility to fosfomycin (Table 2). The fosfomycin resistance was restored by plasmid-borne expression of *AbaF* (in *A. baumannii* *pabaF*). The susceptibility profile for kanamycin could not be determined as insertion of pMo130 resulted in chromosomally encoded kanamycin resistance. However, there was no change in the MIC of the other aminoglycosides tested.

Fosfomycin induces the expression of *abaF*

There was a 2.5-fold and 2.9-fold increase in the expression of *abaF* in *A. baumannii* cells treated with 1×MIC (256 mg/L) and 2×MIC (512 mg/L) fosfomycin, respectively, as compared with the expression of *abaF* in cells that were not treated with fosfomycin (Figure 2a). A further 7-fold increase was observed in the case of cells treated with 4×MIC (1024 mg/L) fosfomycin.

Exposure to fosfomycin results in selection of drug-resistant mutants with increased expression of *abaF*

Growth of *A. baumannii* cells in the presence of increasing amounts of fosfomycin led to isolation of drug-resistant mutants and the expression of *abaF* in cells resistant to 1×, 2× and 4×MIC concentrations was determined by qRT-PCR. It was observed that the expression of *abaF* was about 2.5 times higher ($P<0.02$) in cells resistant to 1×MIC fosfomycin than the cells growing in the absence of fosfomycin (Figure 2b), which increased to about 6 times in cells resistant to 2×MIC fosfomycin ($P<0.01$) and about 14 times in cells resistant to 4×MIC fosfomycin ($P<0.02$). The expression of AbsR25 was low in these fosfomycin-resistant

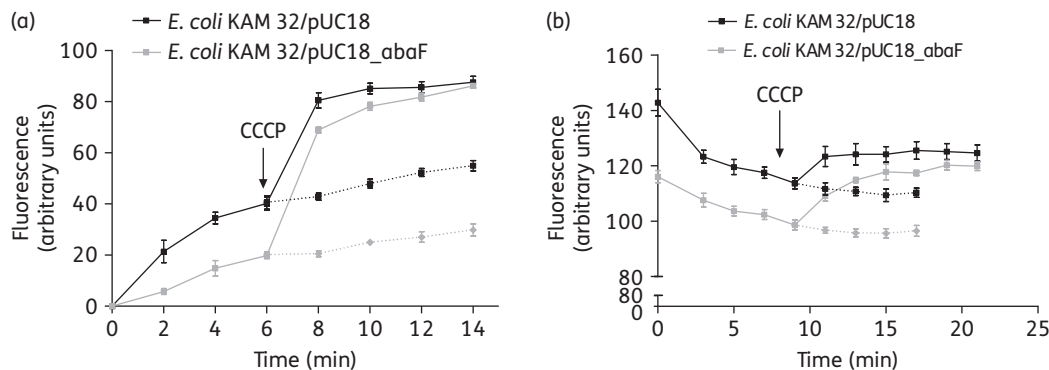


Figure 1. (a) Accumulation of EtBr. EtBr accumulation in *E. coli* KAM32/pUC18 (black squares) and *E. coli* KAM32/pUC18_abaF (grey squares), in the presence of glucose, was determined using a fluorescence plate reader. The efflux inhibitor CCCP was added to the reaction mixture at the timepoint marked by an arrowhead. Each point represents the mean of three different readings with error bars representing the standard deviations ($P<0.05$, Student's *t*-test). (b) Efflux of EtBr. *E. coli* KAM32/pUC18 (black squares) and *E. coli* KAM32/pUC18_abaF (grey squares) cells were incubated in a medium containing EtBr to facilitate loading of the cells with EtBr. Glucose was added to the medium, to a final concentration of 0.4% (w/v), to initiate efflux of EtBr, which was determined by reading the fluorescence of EtBr. The efflux inhibitor CCCP was added to the reaction mixture at the timepoint marked by an arrowhead. Each point represents the mean of three different readings with error bars representing the standard deviations ($P<0.05$, Student's *t*-test). The broken lines represent the relative fluorescence in cases where CCCP was not added at the specified timepoint.

mutants: 0.05-fold (1×MIC, $P<0.001$), 0.15-fold (2×MIC, $P<0.001$) and 0.2-fold (4×MIC, $P<0.001$) as compared with WT *A. baumannii*.

Biofilm formation is affected by disruption of *abaF*

Biofilms are an important characteristic of *A. baumannii* that help the bacterium to survive on inanimate objects and resist the action of various antibiotics. Disruption of *abaF* resulted in reduced biofilm formation (Figure 3a). Defects in biofilm formation were complemented when *abaF* was expressed in a plasmid (in *A. baumannii pabaF*).

AbaF is required for virulence of *A. baumannii* in *C. elegans*

C. elegans worms have been used as a model organism to study the virulence of *A. baumannii*.²¹ It was observed that no worm survived until the 7th day feeding on WT *A. baumannii* cells. However, the last surviving worm feeding on *A. baumannii ΔabaF* cells survived for 11 days (Figure 3b).

Table 2. MICs of various compounds for *A. baumannii* WT, *A. baumannii ΔabaF* and *A. baumannii pabaF*

Compound	MIC (mg/L)		
	<i>A. baumannii</i> WT	<i>A. baumannii</i> <i>ΔabaF</i>	<i>A. baumannii</i> <i>pabaF</i>
Fosfomycin	256	32	256
Chloramphenicol	8	2	—
EtBr	1	4	2
Minocycline	0.125	0.125	0.125
Gentamycin	1	1	1
Tobramycin	1	1	1
Streptomycin	16	16	16
Amikacin	0.5	0.5	0.5
Tetracycline	2	2	2
Nalidixic acid	0.5	0.5	0.5

Clinical strains of *A. baumannii* express *abaF*

Twenty-four clinical strains of *A. baumannii*, resistant to fosfomycin, were analysed for the expression of *abaF*. All but 2 (RPTC 7 and RPTC 21) of the 24 clinical strains of *A. baumannii* tested positive for the expression of *abaF* during the exponential growth phase (Figure 4). The MIC of fosfomycin for clinical strains decreased by a factor of 2-fold (9 strains), 4-fold (7 strains), 8-fold (7 strains) and 16-fold (1 strain) in the presence of efflux inhibitor CCCP (Table S3, available as Supplementary data at JAC Online), suggesting the role of efflux in fosfomycin resistance.

Discussion

A. baumannii is a threat in clinical settings due to its multitude of infections, recalcitrance, natural competence and multiple drug resistance.²³ In our previous report, we identified a novel regulatory small RNA, AbsR25, in *A. baumannii*. It was observed that AbsR25 was involved in negative regulation of a putative efflux pump gene, *abaF*, based on observations in qRT-PCR experiments.¹⁷ This prompted us to study the properties of AbaF and we conducted a series of basic experiments to study the importance of AbaF in *A. baumannii*.

AbaF was designated as an MFS transporter due to the presence of characteristic 12 transmembrane helical domains that are associated with the MFS-type efflux pumps (Figure S1, available as Supplementary data at JAC Online).²⁴ Experimental evidence indicates that AbaF is a transporter that is responsible for efflux of fosfomycin in *E. coli* KAM32. Although a variety of mechanisms of resistance to fosfomycin have been explained, there is no report of efflux-mediated resistance to this antibiotic, which is quite an interesting aspect of this transporter.

The *A. baumannii* cells with disrupted *abaF* were 8-fold more susceptible to fosfomycin (corroborating the results obtained using an overexpression model of *E. coli*) and the MIC value was 32 mg/L, which falls below the EUCAST breakpoint for fosfomycin resistance. Interestingly, the *abaF*-debilitated cells were also 4 times more susceptible to chloramphenicol, an observation that was not evident in *E. coli* KAM32. Chloramphenicol might also be another secondary substrate of AbaF, which may be a subject

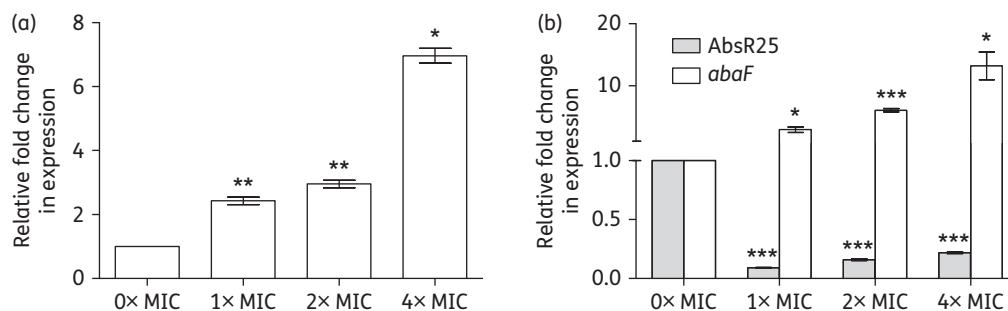


Figure 2. (a) Induction of *abaF* expression by fosfomycin treatment. Actively growing cells of *A. baumannii* ATCC 17978 were treated with increasing concentrations (corresponding to 1×, 2× and 4×MIC) of fosfomycin for 2 h. After treatment, total RNA was isolated and reverse transcribed to cDNA. Change in expression of *abaF* relative to expression in control condition (0×MIC) was determined by qRT-PCR. (b) Expression of AbsR25 and *abaF* in fosfomycin-resistant mutants. Fosfomycin-resistant mutants of *A. baumannii* were selected by serially passaging the WT cells in the presence of increasing concentrations of fosfomycin (corresponding to 1×, 2× and 4×MIC). The total RNA from the mutants was isolated and reverse transcribed to cDNA. Change in expression of AbsR25 (grey bars) and *abaF* (white bars) relative to WT (0×MIC) cells was determined by qRT-PCR. P values were calculated by the paired Student's t -test. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

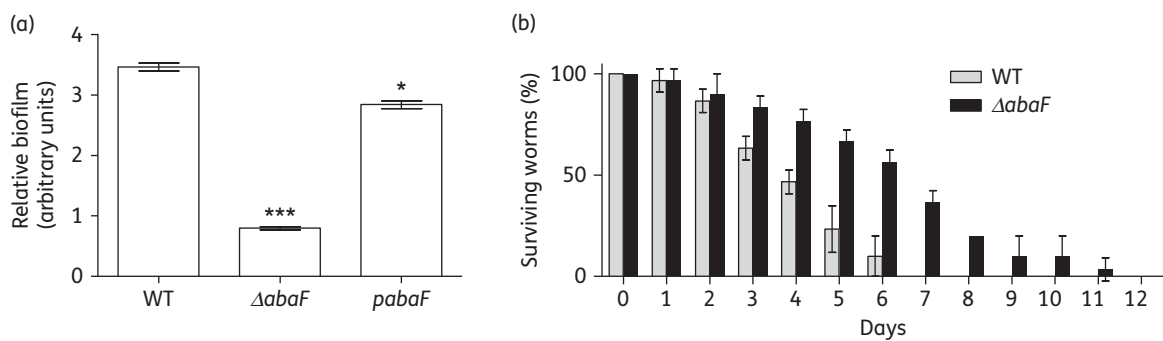


Figure 3. (a) Biofilm formation by WT *A. baumannii*, *A. baumannii* $\Delta abaF$ and *A. baumannii* *pabaF*. Biofilms of WT *A. baumannii* (WT), *A. baumannii* $\Delta abaF$ ($\Delta abaF$) and *A. baumannii* *pabaF* (*pabaF*) formed on the wells of 96-well microtitre plates after 24 h of static incubation at 37°C were stained with 1% crystal violet (CV). The stain picked up by the biomass was dissolved in 95% ethanol and the OD₅₉₅ was determined spectrophotometrically. The relative biofilm was determined as the ratio of the OD₆₀₀ of the cellular biomass (prior to staining) and the OD₅₉₅ of the dissolved stain. The bars represent means of triplicate values with error bars representing standard deviations. (b) Survival of *C. elegans* worms feeding on WT *A. baumannii* and *A. baumannii* $\Delta abaF$. The percentage survival of worms feeding on WT *A. baumannii* (WT, grey bars) and *A. baumannii* $\Delta abaF$ ($\Delta abaF$, black bars) was determined by counting the number of live cells in each plate over the course of time. *P* values were calculated by the paired Student's *t*-test. **P*<0.05; ***P*<0.01; ****P*<0.001.

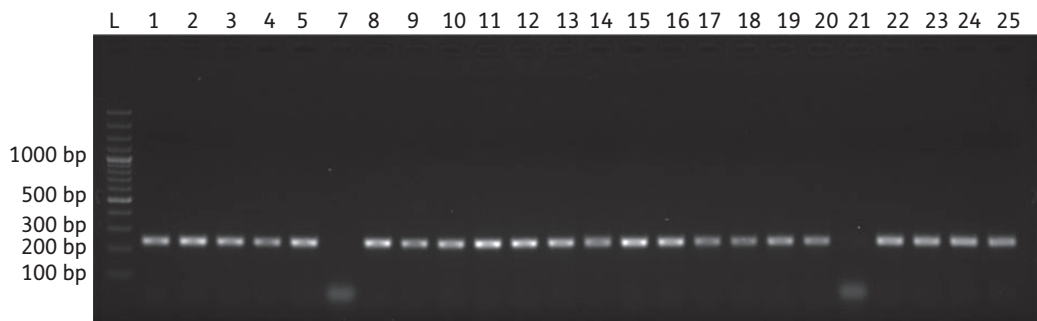


Figure 4. Expression of *abaF* in clinical strains of *A. baumannii*. A 230 bp internal region of *abaF* was amplified from cDNA synthesized from total RNA of 24 clinical strains of *A. baumannii*. The lane numbers represent the designation of the clinical strain (*A. baumannii* RPTC1–RPTC25). Lane L contains 100 bp plus DNA ladder (ThermoScientific, USA).

for future studies. A further direct involvement of AbaF in the active efflux of fosfomycin was suggested by the increase in expression of *abaF* on brief exposure (2 h) to fosfomycin. The increased expression of *abaF* in *A. baumannii* might be assisting the cells in effluxing out the excess of antibiotic and hence promoting cell survival. In the case of a prolonged exposure to fosfomycin, *A. baumannii* cells that were resistant to the antibacterial action of fosfomycin were obtained. These resistant mutants selected in the presence of high amounts of fosfomycin also showed increased expression of *abaF*. The *A. baumannii* cells overexpressing the fosfomycin resistance determinant, *abaF*, had a clear advantage and therefore were selected over the others. In line with our previous observations, the expression of AbsR25 in the cells overexpressing *abaF* was low. However, the expression of AbsR25 was not inversely related to the change in expression of *abaF*, which indicates that AbsR25 has targets other than *abaF*.

The importance of AbaF is highlighted by the fact that it is constitutively expressed in most, if not all, of the clinical strains of *A. baumannii* during active growth. However, it is quite interesting to note that despite expressing *abaF*, not all the clinical strains were sensitized to fosfomycin on inhibition of efflux by CCCP. This observation hints that efflux, though an important reason,

might not be the only mechanism responsible for fosfomycin resistance. There must be other fosfomycin resistance mechanisms active in these clinical strains that did not show any significant change in the MIC of fosfomycin in the presence of CCCP. However, this should not undermine the role of efflux-mediated resistance to fosfomycin. It might be a case similar to fluoroquinolone resistance, which is mediated by efflux as well as gyrase mutations.²⁵

It is surprising that despite a general consensus that fosfomycin is not active against *A. baumannii*, there is no concrete evidence in the literature explaining the reasons for this. Recently, the synergistic activity of fosfomycin with colistin, minocycline and polymyxin B against MDR *A. baumannii* has revived interest in fosfomycin as a drug of choice.^{14,26} However, the high frequency of mutant selection and adaptations leading to increased expression of *abaF* (and increased efflux of fosfomycin thereof), as per our observations, could compromise the success of these combinatorial therapeutics.

Some more interesting findings from disruption of *abaF* in *A. baumannii* were impaired biofilm formation and decreased virulence. Since its disruption resulted in reduced biofilm formation, AbaF might also be one of the efflux pumps that are involved in

extrusion of biofilm material. Such efflux pumps have been reported to be involved in biofilm formation in *E. coli*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Staphylococcus aureus* and *Klebsiella* strains.^{27–30} Efflux pump inhibition and disruption has been implicated in decreased virulence owing to the proposed capacity of efflux pumps to expel host-derived antibacterial factors.³¹ AbaF also seems to play a similar role in *A. baumannii*, which is evident by the markedly high survival of *C. elegans* on *A. baumannii* cells with disrupted *abaF*. Although some efflux pumps have been implicated in coping with other stresses, such as oxidative and nitrosative stress,³² AbaF does not seem to play any role in these conditions as there was no difference in response of WT as well as *abaF*-debilitated cells to these stresses (data not shown).

Overall, this study provides new insight into the resistance of *A. baumannii* to a drug, fosfomycin, which has been the subject of renewed interest. The MFS transporter AbaF actively effluxes out fosfomycin, rendering the cells resistant, and its expression is upregulated on exposure to fosfomycin. AbaF is expressed in fosfomycin-resistant clinical strains of *A. baumannii* and these strains are rendered susceptible to fosfomycin in the presence of efflux inhibitors. AbaF is also involved in the secretion of biofilm matrix that contributes to the pathogenicity of the bacterium and might be involved in expulsion of host defence molecules, resulting in a significant impact on the virulence of *A. baumannii*.

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Transparency declarations

None to declare.

Supplementary data

Supplementary methods, Figure S1 and Tables S1–S3 are available as Supplementary data at JAC Online (<http://jac.oxfordjournals.org/>).

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