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Regulation of the AcrAB efflux system by the quorum-sensing regulator AnoR in *Acinetobacter nosocomialis*[§]

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Multidrug efflux pumps play an important role in antimicrobial resistance and pathogenicity in bacteria. Here, we report the functional characterization of the RND (resistance-nodulation-division) efflux pump, AcrAB, in *Acinetobacter nosocomialis*. An *in silico* analysis revealed that homologues of the AcrAB efflux pump, comprising AcrA and AcrB, are widely distributed among different bacterial species. Deletion of *acrA* and/or *acrB* genes led to decreased biofilm/pellicle formation and reduced antimicrobial resistance in *A. nosocomialis*. RNA sequencing and mRNA expression analyses showed that expression of *acrA/B* was downregulated in a quorum sensing (QS) regulator (*anoR*)-deletion mutant, indicating transcriptional activation of the *acrAB* operon by AnoR in *A. nosocomialis*. Bioassays showed that secretion of N-acyl homoserine lactones (AHLs) was unaffected in *acrA* and *acrB* deletion mutants; however, AHL secretion was limited in a deletion mutant of *acrR*, encoding the *acrAB* regulator, AcrR. An *in silico* analysis indicated the presence of AcrR-binding motifs in promoter regions of *anoI* (encoding AHL synthase) and *anoR*. Specific binding of AcrR was confirmed by electrophoretic mobility shift assays, which revealed that AcrR binds to positions -214 and -217 bp upstream of the translational start sites of *anoI* and *anoR*, respectively, demonstrating transcriptional regulation of these QS genes by AcrR. The current study further addresses the possibility that AcrAB is controlled by the osmotic stress regulator, OmpR, in *A. nosocomialis*. Our data demonstrate that the AcrAB efflux pump plays a crucial role in biofilm/pellicle formation and antimicrobial resistance in *A. nosocomialis*, and is under the transcriptional control of a number of regulators. In addition, the study emphasizes the interrelationship of QS and AcrAB efflux systems in *A. nosocomialis*.

Keywords: *Acinetobacter nosocomialis*, efflux pump, quorum sensing, AcrAB

Introduction

Multidrug efflux pumps, which are present in almost all bacterial species, are determinants of antibiotic resistance that function by extruding drugs. These efflux pumps are distributed in five major families, with members of the resistance-nodulation-division (RND) transporter family being responsible for antibiotic resistance in Gram-negative bacteria (Piddock, 2006a). RND efflux pumps were first reported independently in *Escherichia coli* (AcrAB-TolC) (Ma *et al.*, 1993, 1995) and *Pseudomonas aeruginosa* (MexAB-OprM) (Poole *et al.*, 1993). As is the case for other RND efflux pumps, the AcrAB-TolC efflux pump comprises a transporter (efflux) protein, AcrB, in the inner membrane; an outer membrane protein channel, TolC; and a periplasmic accessory protein, AcrA (Koronakis *et al.*, 2004). In addition to *E. coli* and *P. aeruginosa*, many other bacterial strains are reported to express AcrAB-TolC efflux pumps or their homologues, including *Salmonella* Typhimurium, *Klebsiella aerogenes*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Campylobacter* spp., and *Borrelia burgdorferi* (Poole *et al.*, 1993; Pumbwe *et al.*, 2004; Buckley *et al.*, 2006; Bunikis *et al.*, 2008). AcrAB-TolC efflux pumps are known to recognize and transport a wide range of compounds, including antibiotics, bile salts, dyes, and detergents (Nikaido, 1996). In *E. coli*, the AcrAB-TolC efflux pump is a housekeeping pump that confers resistance to chloramphenicol, fluoroquinolone, tetracycline, novobiocin, rifampin, fusidic acid, nalidixic acid, and β-lactam antibiotics (Piddock, 2006b).

In addition to their role in antibiotic resistance, RND efflux pumps are involved in bacterial pathogenicity by directly contributing to bacterial colonization and persistence in their ecological niche (Piddock, 2006b). It has been reported that *acrB* and *tolC* gene mutants of *S.* Typhimurium poorly colonize the avian gut, indicating the involvement of the AcrAB-TolC system in pathogenesis (Buckley *et al.*, 2006). MexAB-OprM, the AcrAB-TolC homologue in *P. aeruginosa*, has been reported to export virulence determinants, and mutants lacking MexAB-OprM are unable to invade Madin-Darby canine kidney (MDCK) cells (Hirakata *et al.*, 2002). In addition to their direct role in bacterial pathogenesis, RND efflux pumps

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contribute to bacterial virulence indirectly by altering the quorum-sensing (QS) response in bacteria. The QS system is mediated by secreted chemical signaling molecules called autoinducers, which must be transported across the cell membrane. For example, in *P. aeruginosa*, acyl homoserine lactones (AHLs), which act as QS signals, are exported by the MexAB-OprM system; thus, overexpression of the Mex pump leads to reduced virulence owing to increased efflux of AHLs (Evans *et al.*, 1998).

Among *Acinetobacter* species, RND efflux pumps are almost exclusive to *A. baumannii*, *A. pittii*, and *A. nosocomialis*, but their orthologues are distributed among other bacterial strains, such as *E. coli* and *P. aeruginosa*. Three efflux systems–AdeABC (Magnet *et al.*, 2001), AdeFGH (Coyne *et al.*, 2010), and AdeIJK (Damier-Piolle *et al.*, 2008)–are the primary contributors to multidrug resistance (MDR) in *A. baumannii*. AdeABC, the first characterized RND efflux pump in *A. baumannii*, is composed of the major fusion protein AdeA, the multidrug transporter AdeB, and the outer membrane factor OMF (Magnet *et al.*, 2001). AdeA and AdeB show similarity to AcrA (55%) and AcrB (68%), respectively, of *E. coli*, and to MexA (58%) and MexB (67%) of *P. aeruginosa*. Overexpression of the AdeABC efflux pump confers resistance to aminoglycosides, β-lactams, fluoroquinolones, tetracyclines, tigecycline, macrolides, chloramphenicol, and trimethoprim (Magnet *et al.*, 2001). The AdeFGH efflux pump, which is controlled by the transcriptional regulator AdeL (Coyne *et al.*, 2010), contributes to high-level resistance to fluoroquinolones, chloramphenicol, trimethoprim and clindamycin, and decreased susceptibility to tetracyclines, tigecycline and sulfamethoxazole, but has no effect on resistance to β-lactams or aminoglycosides. The AdeIJK efflux pump provides resistance to β-lactams (e.g., ticarcillin, cephalosporins, and aztreonam), fluoroquinolones, tetracyclines, tigecycline, lincosamides, rifampin, chloramphenicol, cotrimoxazole, novobiocin, and fusidic acid (Damier-Piolle *et al.*, 2008). A recent report identified an RND efflux system comprising the membrane protein ArpA and the transmembrane protein ArpB in *A. baumannii* AB5075 showing that the genetic organization of the *arpAB* efflux operon is similar to that of *acrAB* in *E. coli* and *mexAB* in *P. aeruginosa*. This report further showed that the ArpAB efflux system is involved in the opaque-to-translucent switch and confers resistance to aminoglycosides, such as amikacin and tobramycin.

We previously reported a functional characterization of the TetR-type transcriptional regulator, AcrR, which controls an as-yet-uncharacterized AcrAB efflux pump in the opportunistic nosocomial pathogen *A. nosocomialis* (Subhadra *et al.*, 2018). We further demonstrated that AcrR regulates several phenotypes, including motility, biofilm/pellicle formation and pathogenesis, in *A. nosocomialis*. In the current study, we functionally characterized the AcrAB efflux pump and demonstrated its interrelationship with the QS system in *A. nosocomialis*. To the best of our knowledge, this is the first study to report the characterization of an AcrAB efflux pump in an *Acinetobacter* strain.

Materials and Methods

Bacterial strains, plasmids, and culture conditions

Table 1 depicts the bacterial strains and plasmids used in this study. *E. coli* strains were cultivated in Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) at 37°C, while the *A. nosocomialis* strains were grown at 30°C or 37°C in either LB or Mueller Hinton (MH) media (Difco). Whenever required, the media was supplemented with antibiotics for *E. coli* (kanamycin, 50 μg/ml; chloramphenicol, 20 μg/ml) as well as *A. nosocomialis* (kanamycin, 30 μg/ml; ampicillin, 100 μg/ml). The bioassay reporter strain, *Agrobacterium tumefaciens* NT1 (pDCI41E33) was grown in defined minimal medium at 30°C (Zhang *et al.*, 1993). The bioassay plate for the detection of AHLs using the reporter strain was prepared as previously described (Park *et al.*, 2003).

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference or source
ATCC 17903	Type strain	ATCC
Δ*acrR*	*acrR* deletion mutant of ATCC 17903	Subhadra *et al.* (2018)
Δ*anoR*	*acrR* deletion mutant of ATCC 17903	Oh and Choi (2015)
Δ*acrA*	*acrR* deletion mutant of ATCC 17903	This study
Δ*acrB*	*acrR* deletion mutant of ATCC 17903	This study
E. coli DH5α	F- *thi-I end A1 hsdR17* (r^-m^-) *supE44* Δ*lacU169* (Φ80*lacZ*ΔM15) *recA1 gyrA96 relA*	Hanahan (1983)
E. coli DH5α λ *pir*	*supE44* △*lacU169*(ϕ80 *lacZ*△M15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1 λpir* (phage lysogen); plasmid replication	Laboratory collection
E. coli S17-1 λ *pir*	*λpir* lysogen; *thi pro hsdR hsdM*$^+$ *recA* RP4-2 Tc::Mu-Km::Tn7; Tp^r Sm^r ; host for π-requiring plasmids; conjugal donor	Simon *et al.* (1983)
E. coli BL21(DE3)	*ompT hsdS*$_B$($r_B^-m_B^-$) *gal dcm* (DE3)	Studier and Moffatt (1986)
pUC4K	pUC4 with *nptI*; Ap^r, Km^r	Amersham Pharmacia Biotech
pHKD01	pDS132, multicloning sites; *ori*R6K *sacB*; Cm^r	Oh *et al.* (2015)
pOH2001	pHKD01 with Δ*acrA*::*nptI*; Cm^r, Km^r	This study
pOH2002	pHKD01 with Δ*acrB*::*nptI*; Cm^r, Km^r	This study
pET-*acrR*	pET_28a carrying the coding region of *acrR*	Subhadra *et al.* (2018)
pET-*ompR*	pET_28a carrying the coding region of *ompR*	This study

[a] Tp^r, trimethoprim resistant; Sm^r, streptomycin resistant; Ap^r, ampicillin resistant; Km^r, kanamycin resistant; Cm^r, chloramphenicol resistant.

Table 2. Oligonucleotides used in this study

Oligonucleotides	Sequence (5′→3′)[a,b]	Purpose
AcrA01F	CAGCTTGACCATCACGTGC	Construction of *acrA* deletion mutant
AcrA01R	CCATTCCGAGAGATTGAATTTCTTATCGCTCTTATTCAAACTGATT	Construction of *acrA* deletion mutant
AcrA02F	AATTCAATCTCTCGGAATGGG	Construction of *acrA* deletion mutant
AcrA02R	ACCTTCTTCACGAGGCAGACCCGACATCGAATACATTCATGC	Construction of *acrA* deletion mutant
AcrB01F	AACCTCTCATCATGAGTATGGTGA	Construction of *acrB* deletion mutant
AcrB01R	TCACAAAAAATTTATGTATTAAAATAAAGGACGATACCTTTATTGTT	Construction of *acrB* deletion mutant
AcrB02F	AATACATAAATTTTTTGTGAATAAAGGTG	Construction of *acrB* deletion mutant
AcrB02R	ACCTTCTTCACGAGGCAGAC ACCATCACACACGTCACATGG	Construction of *acrB* deletion mutant
nptIF	GTCTGCCTCGTGAAGAAGGTG	Amplification of *nptI*
nptIR	GATCCGTCGACCTGCAGG	Amplification of *nptI*
PET_*ompR*_F	*GGATCC*GATGAGTTTAGTTGTACCTGC	For overexpression of OmpR
PET_*ompR*_R	*AAGCTT*TTCAGCACCATCTGGAACAA	For overexpression of OmpR
P*anoI*_F	GACTGGGAATTCGTTGAAC	For EMSA
P*anoI*_R	CACTACAAGTGCTTCCACT	For EMSA
P*anoR*_F	CAAGCATAATCTGTATATTGAA	For EMSA
P*anoR*_R	AGTTATCTCTTGTGAATCCAA	For EMSA
P*acrAB*_F	AAAACCCTCTAATAAAAGATTAA	For EMSA
P*acrAB*_R	ATCTTATCGCTCTTATTCAAA	For EMSA
16S rDNA_F	CGTGCTACAATGGTCGGT	For EMSA
16S rDNA_R	GTATTCACCGCGGCATTC	For EMSA
16S_F	AAGACTAAAACTCAAATGAA	For qRT-pCR
16S_R	TGGAAAGTTCTTACTATGTC	For qRT-pCR
AcrA_F	GATGGTAACGCAGCCTTC	For qRT-pCR
AcrA_R	GCCGTAACTTGTCCACCT	For qRT-pCR
AcrB_F	AGCGACTGTTGTTGGCGA	For qRT-pCR
AcrB_R	TACCAGCATTGACCG AAC	For qRT-pCR

[a] Regions of oligonucleotides that are not complementary to the corresponding templates are underlined.
[b] Added restriction site sequences are indicated in italics.

DNA manipulations

Standard molecular cloning procedures were adopted in this study (Sambrook *et al.*, 1989). The oligonucleotides used in this study were purchased from Macrogen Co., Ltd. and are listed in Table 2. PureHelix™ Genomic DNA Prep Kit (Nanohelix Co. Ltd.) and AccuPrep® Plasmid Extraction Kit (Bioneer Corp.) were used for isolating chromosomal DNA from *A. nosocomialis* and plasmid DNA from *E. coli*, respectively. DNA fragments were amplified by polymerase chain reaction (PCR) using PrimeSTAR GXL Taq DNA polymerase (TaKaRa). The amplified DNA fragments were purified or eluted from gel using HiGene™ Gel & PCR Purification System (BioFact Co., Ltd.). The plasmids were introduced into *E. coli* and *A. nosocomialis* by heat shock method (Hanahan, 1983) and conjugation (Oh *et al.*, 2015), respectively. All the restriction and DNA modifying enzymes were purchased from New England Biolabs. The mutants and recombinant strains constructed were confirmed by sequencing (Macrogen Co., Ltd.).

In silico analysis

Bioinformatic analysis was performed using the *A. nosocomialis* ATCC 17903 (NCTC 8102) genome sequence retrieved from GenBank (GenBank accession no. CP029351). Phylogenetic analysis was carried out to determine the relationship of *acrA* and *acrB* of *A. nosocomialis* with that of other related strains. The program MEGA 6.06 (Tamura *et al.*, 2013) was used for the construction of the phylogenetic tree based on Maximum Likelihood approach. In order to identify the binding motif of AcrR in the promoter region of *anoR* and *anoI* (P*anoR* and P*anoI*) of *A. nosocomialis*, multiple sequence alignment was performed with the P*anoR* and P*anoI* and the known AcrR binding sequence from *E. coli* (Su *et al.*, 2007). Similarly, multiple sequence alignment was carried out with the *acrAB* promoter region (P*acrAB*) and the known binding motifs of OmpR (Raczkowska *et al.*, 2015) in order to check the binding of OmpR to P*acrAB*.

RNA sequencing and analysis

The total RNA was isolated from *A. nosocomialis* wild type and *anoR* deletion mutant using High Pure RNA Isolation Kit (Roche Diagnostics GmbH) after cultivating the cells in LB until stationary phase. The isolated RNA was stored at -80°C until use. All RNA-sequencing and transcriptome alignment procedures were conducted by ChunLab. The RNA was subjected to a subtractive Hyb-based rRNA removal process using the MICROBExpress Bacterial mRNA Enrichment Kit (Ambion). The library construction was performed as described earlier (Li and Nikaido, 2004). RNA sequencing was performed using 2 runs of the Illumina HiSeq and the genome sequence of *A. nosocomialis* was retrieved from the NCBI database (accession number CP029351). CLC Geno-

mics Workbench 6.5.1 tool (CLC bio) was used to align the quality-filtered reads and the mapping was based on a minimal length of 100 bp, with an allowance of up to 2 mismatches. The mapped data was normalized with the Relative Log Expression (RLE) method and visualized using the CLRNAseq program (ChunLab). The genes that showed a fold change larger than 2.0 and lower than 0.5 were considered as up- and down-regulated genes respectively.

Overexpression and purification of His_6-OmpR

For the overexpression of hexahistidyl-tagged OmpR (His_6-OmpR), the complete coding region of *ompR* (765 bp) of *A. nosocomialis* was amplified by PCR and subcloned into His-tag expression vector, pET22b yielding pET-*ompR*. The resultant recombinant plasmid was introduced into *E. coli* strain BL21(DE3) by transformation. The cells harboring the recombinant plasmid, pET-*ompR* was grown in LB at 37°C until OD_{600} 0.5 and the cells were then induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The induced culture was further incubated for 5 h at 30°C, harvested by centrifugation and lysed by sonication (Vibra-Cell™, Sonics & Materials, Inc.) in lysis buffer (20 mM Tris-HCl; pH 8, 500 mM NaCl, and 5 mM imidazole). After centrifugation, the OmpR protein in the soluble supernatant was purified by nickel–nitrilotriacetic (Ni-NTA) affinity chromatography according to the manufacturer's (Qiagen) protocol. The purified OmpR was dialyzed overnight against phosphate buffered saline (PBS) to remove imidazole and then directly used for promoter binding assays.

Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assay was performed to check the binding of AcrR and OmpR to the promoter regions of *anoR*/*anoI* and *acrAB*, respectively, based on the protocol depicted earlier (Alves and Cunha, 2012). The promoter fragments (P*anoR*, 272 bp; P*anoI*, 303 bp; P*acrAB*, 148 bp) and 16S rDNA of 149 bp were amplified by PCR and binding assay was carried out with His_6-AcrR and His_6-OmpR as described earlier (Subhadra *et al.*, 2018). In the case of AcrR, 200 ng of the promoter fragment was incubated with varying concentrations of purified His_6-AcrR (0 to 1.0 µg) in 15 µl of binding buffer (250 mM phosphate buffer; pH 7.5, containing 50 mM NaCl, 500 mM KCl, 10 mM dithiothreitol, 10 mM ethylenediaminetetraacetic acid, 3 µg bovine serum albumin and 1 µg Poly[d{I-C}]). The binding assay was carried out at room temperature (RT) for 30 min followed by electrophoresis in 6% prerun (20 min at 80 V) native polyacrylamide gel in 1× Tris/Borate/EDTA (TBE) buffer for 1 h at 80 V. In the case of OmpR, the binding buffer contained 40 mM Tris-HCl (pH 8.0), 100 mM KCl, 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 3 µg bovine serum albumin and 5% glycerol. The reactions were then analyzed by electrophoresis on 0.7% prerun (30 min at 50 V) agarose gel in 0.25× TBE for 1 h at 50 V. The binding specificity of AcrR and OmpR was confirmed by including 16S rDNA in the EMSA reaction mixture instead of the promoter fragment. The gels were subsequently removed and stained with ECOred (MOSAICON) for visualization.

RNA extraction and real-time quantitative reverse transcription PCR (RT-PCR)

A. nosocomialis strains were cultivated in LB medium until the stationary phase (OD_{600}: 1.5) and total RNA was isolated with High Pure RNA Isolation Kit (Roche Diagnostics GmbH) according to the manufacturer's protocol. One microgram of DNase-treated total RNA was used for the synthesis of complementary DNA (cDNA) with Reverse Transcription Master Premix (ELPIS Biotech. Inc.) per manufacturer's instructions. Quantitative RT-PCR was performed in triplicates using StepOnePlus™ Real-Time PCR System (Applied Biosystems) with Power SYBR® Green PCR Master Mix (Applied Biosystems). A 16S rRNA was considered as a reference and gene expression was normalized by the $\Delta\Delta$CT method (Livak and Schmittgen, 2001).

Construction of deletion mutants of *acrA* and *acrB*

Markerless gene deletion mutants of *acrA* and *acrB* were constructed as described previously (Oh *et al.*, 2015). To construct the deletion mutant of *acrA*, two DNA fragments covering the upstream and downstream regions of *acrA* were amplified by PCR using primer pairs: AcrA01F and AcrA01R, AcrA02F and AcrA02R. In the case of *acrB* mutant construction, the DNA fragments covering the upstream and downstream regions of *acrB* were amplified using the oligonucleotide pairs: AcrB01F and AcrB01R, AcrB02F and AcrB02R. In addition, a kanamycin resistance gene, *nptI* was amplified from pUC4K using the primers, U1 and U2. The upstream and downstream fragments, and the *nptI* were annealed together by overlap extension PCR using the primers AcrA01F/U2 and AcrB01F/U2 for *acrA* and *acrB*, respectively. The resultant PCR product was ligated into *Fsp*I-digested pHKD01 to result in pOH2001 (for *acrA*) or pOH2002 (for *acrB*) which were then transformed into *A. nosocomialis* by conjugation as described earlier (Oh *et al.*, 2015). The integration of pOH2001 or pOH2002 into the chromosome was confirmed by growing cells on LB plate containing ampicillin and kanamycin. The deletion mutants were screened by growing kanamycin-resistant colonies overnight in LB plate containing 10% (w/v) sucrose. A sucrose-resistant and kanamycin sensitive cell (deletion mutant) was selected and the double crossover event was confirmed by PCR.

Biofilm/pellicle formation assay

Biofilm and pellicle formation assays were carried out based on previously reported protocols with slight modifications (Lee *et al.*, 2008; Giles *et al.*, 2015). For biofilm and pellicle formation assays, *A. nosocomialis* wild type, *acrA* and *acrB* deletion mutants were cultivated in MH broth at 37°C. Twenty-five microliters of the overnight cultures (OD_{600} of 1) were added to 5 ml of MH broth in polystyrene tubes (Ø 17 mm × H 100 mm) (SPL Life Sciences) and the tubes were incubated at different time periods at 30°C without shaking. For biofilm assay, planktonic cells were removed from the cultures and the tubes were washed thrice with sterile distilled water. For pellicle assay, the pellicle was removed and resuspended in 1 ml of PBS after the careful addition of 100% ethanol to the bottom of the pellicle. The pellicle suspension was then centrifuged at 13,000 rpm and the supernatant was carefully

removed. 0.1% (wt/vol) crystal violet (Sigma) was used to stain both biofilm and pellicle for 15 min at RT. Both biofilm and pellicle were washed thrice with sterile distilled water and air dried at RT. The stain absorbed to biofilm or pellicle was eluted with 30% acetic acid and quantified by measuring at OD_{595}. The pellicle/biofilm assay was carried out in quintuplicates at three different occasions.

Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) of antimicrobial agents were determined by either E-test method according to the manufacturer's instructions or by macrodilution method. The antimicrobial agents for the E-test included aztreonam, ceftazidime, ciprofloxacin, tigecycline, colistin, gentamicin, tetracycline, trimethoprim, imipenem, and tobramycin (bioMérieux). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains. An efflux pump inhibitor, phenylalanine-arginine β-naphthylamide (PAβN) (Sigma-Aldrich) was added to Mueller-Hinton agar plates at a concentration of 20 μg/ml. Interpretation of antimicrobial susceptibility was based on the guidelines of the Clinical Laboratory Standards Institute (CLSI, 2017). The critical difference in resistance (CDR) between two groups was defined as fold-changes in MICs of the antimicrobial agents that were ≥ 2 or ≤ 0.5 between the wild type strain and its *acrA* or *acrB* mutant counterparts. The MIC of antibiotic acriflavine was performed by the macrodilution method (CLSI, 2018). The overnight cultures were diluted to an OD_{600} of 0.063 (McFarland standard 0.5) and acriflavine (1 mg/ml stock) was serially diluted (1.95–250 μg/ml) in 1 ml of the diluted cultures. The cultures were in-

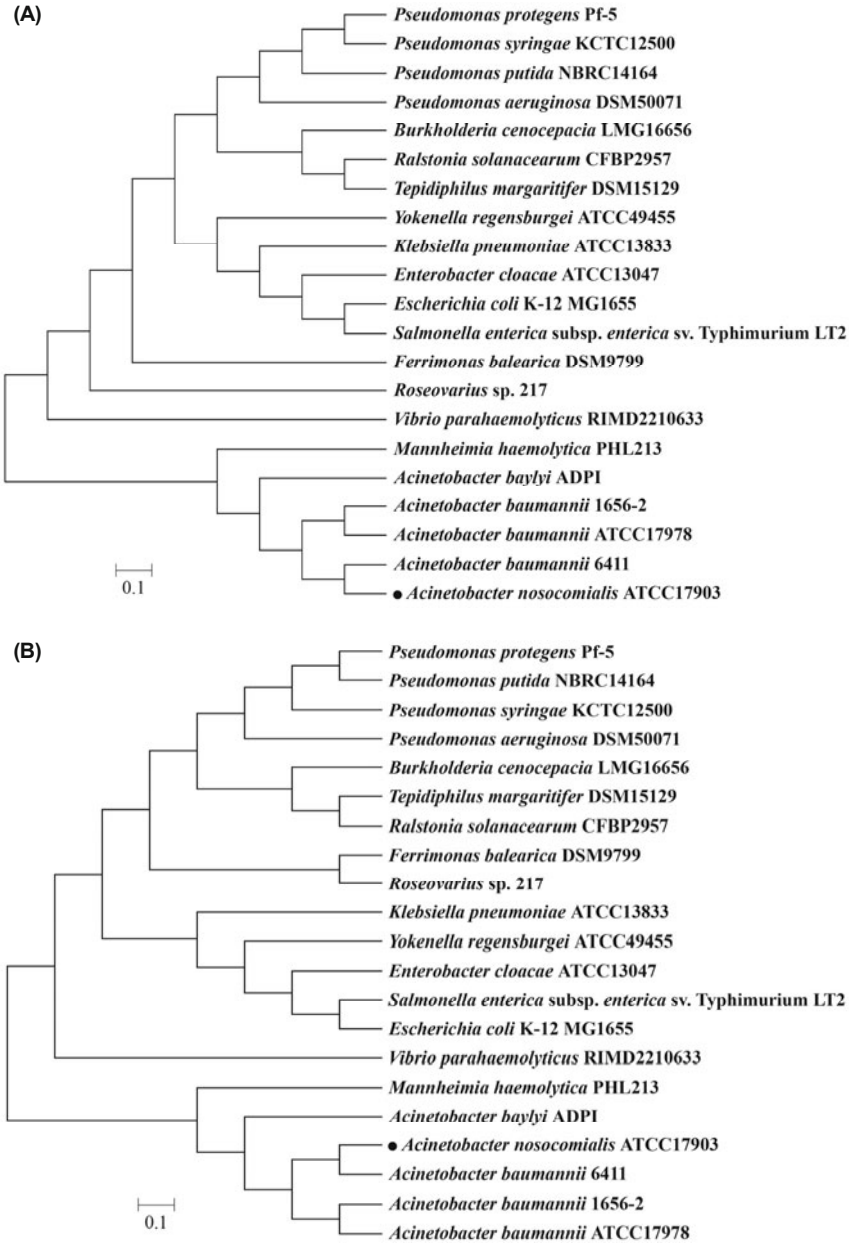

Fig. 1. *In silico* analysis of the *acrAB* operon. Phylogenetic analysis of *acrA* (A) and *acrB* (B) from *A. nosocomialis* in relation to homologues from other bacterial strains. The tree was constructed using MEGA 6.06 based on the maximum-likelihood approach.

cubated for 24 h followed by serial dilution and CFU (colony-forming unit) counting. Antimicrobial susceptibility testing was performed in three independent experiments.

Ethidium bromide accumulation assay

The ethidium bromide (EtBr) accumulation assay was carried out as described previously with slight modifications (Viveiros *et al.*, 2008). Briefly, *A. nosocomialis* wild type and mutant strains were cultivated in LB medium overnight. The bacteria were then centrifuged and resuspended in PBS and the OD_{600} of the culture was adjusted to 0.2. The baseline fluorescence of the culture was monitored with a fluorescence spectrophotometer (Fluoroskan Ascent; Thermo Electron Corporation) at an excitation and emission wavelengths 485 and 590, respectively. EtBr was added to the cultures at a final concentration of 50 μM and the change in fluorescence was measured for 30 min at RT.

Bioassay for the detection of autoinducers

A. nosocomialis wild type and the *acrA* and *acrB* deletion mutants were cultivated overnight in MH at 30°C and then diluted to an OD_{600} of 1. Five microliters of the diluted samples were spotted onto the previously prepared chromoplate overlaid with *A. tumefaciens* NT1 (pDCI41E33) and allowed it to dry for 30 min. The experiments were done in triplicates and the plates were incubated at 30°C for 22 h. As control, 40 μM of N-decanoyl-DL-homoserine lactone N-(3-hydroxydodecanoyl)-L-homoserine lactone (OH-dDHL) was spotted onto overlay plate. The AHL production of the wild type and the mutants was compared based on the diameter of the color zone surrounding the bacterial spots in the chromoplates.

Statistical analysis

Whenever applicable, the experiments were repeated at least three times with consistent results. The significance of difference between two groups was determined by unpaired Student's *t*-test and that among more than three groups was evaluated with one-way ANOVA followed by Tukey's multiple comparison test using statistical software GraphPad Prism v5.01 (GraphPad Software Inc.). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ were considered statistically significant.

Results

In silico analysis of *acrA* and *acrB*

In our previous study, we reported that *acrAB* is a polycistronic operon encoding the AcrAB efflux pump that is under functional regulation by AcrR (Subhadra *et al.*, 2018). The *acrA* and *acrB* genes are widely distributed among different bacterial strains. To understand the relationships of *acrA* and *acrB* with their corresponding homologues, we carried out a phylogenetic analysis. The *acrA* and *acrB* genes from *Acinetobacter* strains showed close similarity to those from *Mannheimia haemolytica* PHL213 and *Vibrio parahaemolyticus* RIMD 2210633 (Fig. 1). At the protein level, AcrA and AcrB proteins share high similarity with the corresponding proteins, AcrA and AcrB from *A. baumannii* 17978 (96.36% and 98.4% for AcrA and AcrB, respectively) and *A. baumannii* 6411 (99.18% and 99.9%), and lower similarity with those from *E. coli* (23.29% and 25.36%), *P. putida* NBRC 14164 (46.78% and 67.58%) and *S.* Typhimurium (27.27% and 24.98%) (Supplementary data Fig. S1A and B).

Construction of deletion mutants and assessment of their growth curves

We next constructed deletion mutants of *acrA* and *acrB* from *A. nosocomialis* ATCC 17903 using the double-crossover method, as detailed in 'Materials and Methods.' Deletion of both genes was confirmed by PCR (Fig. 2A) followed by sequencing. The growth of *acrA* and *acrB* mutants was characterized and compared with that of wild type *A. nosocomialis* by cultivating in LB and measuring the optical density of cultures at 600 nm (OD_{600}) over a 24-h incubation period. The OD_{600} was measured after diluting the cultures and the measured absorbance value was multiplied with the dilution factor. These experiments showed that the growth phenotypes of both *acrA* and *acrB* mutants were similar to that of wild type (Fig. 2B).

Deletion of *acrA* and/or *acrB* decreases biofilm/pellicle formation

It is widely known that multidrug efflux pumps play an important role in biofilm/pellicle formation (Lynch *et al.*, 2007; Kvist *et al.*, 2008). To assess the effect of *acrAB* deletion on

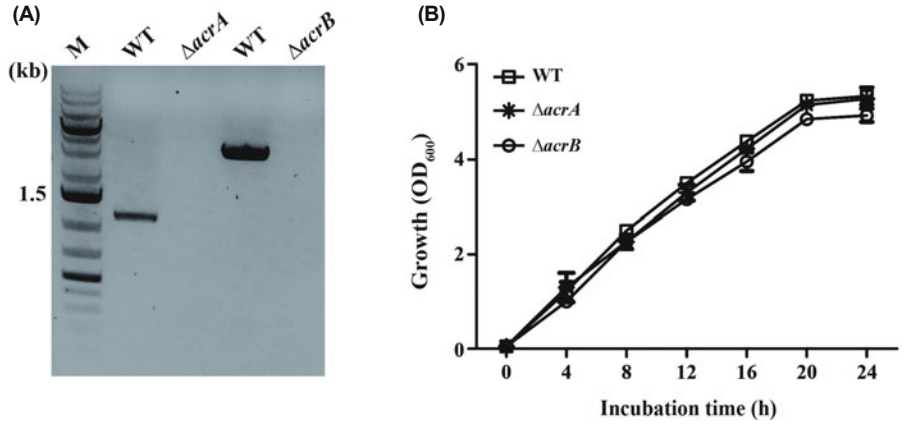

Fig. 2. Confirmation of *acrA* and *acrB* deletion by PCR and characterization of the growth phenotypes of *acrA*- and *acrB*-deletion mutants. (A) The *acrA* and *acrB* mutants were verified by PCR using genomic DNA as a template. WT, wild type; Δ*acrA*, *acrA* mutant; Δ*acrB*, *acrB* mutant; M, DNA molecular weight marker. (B) Growth phenotypes of *acrA* and *acrB* mutants, grown in MH broth for 24 h. The growth phenotypes of wild type and *acrA* and *acrB* mutants are indicated by squares, stars, and circles respectively. Values are means ± SD ($n = 3$).

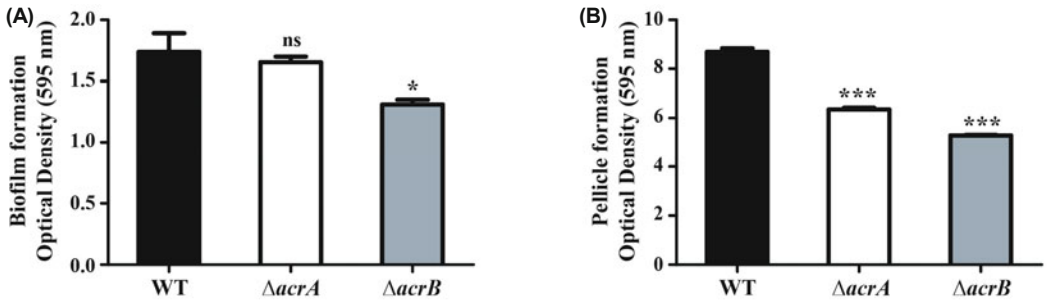

Fig. 3. Increased biofilm/pellicle formation in *acrA*- and/or *acrB*-deletion mutants. Strains were cultivated in MH medium at 30°C, and biofilm/pellicle formation was assessed using a crystal violet staining method. (A) Biofilm formation by *A. nosocomialis* wild type (WT) and *acrA* and *acrB* mutants (Δ*acrA* and Δ*acrB*) at 48 h. (B) Pellicle formation in wild type (WT) and *acrA* and *acrB* mutants (Δ*acrA* and Δ*acrB*) at 72 h. Values are means ± SD ($n = 5$; *$P < 0.05$ and *** $P < 0.001$ for mutants vs. wild type). Biofilm/pellicle assays were performed on three separate occasions.

biofilm/pellicle formation by *A. nosocomialis*, we cultivated wild type and deletion mutants in MH broth at 30°C for 72 h, and separately quantified the biofilm attached to the culture tubes (48 h) and the pellicle formed at the air-liquid interface (72 h) using a crystal violet staining method. The *acrB* mutant displayed decreased biofilm formation compared to the wild type (Fig. 3A). Pellicle formation was also decreased in both mutants, although the *acrB* mutant was more adversely affected than the *acrA* mutant (Fig. 3B). These results indicate that the AcrAB efflux pump is important for biofilm/pellicle formation in *A. nosocomialis*.

Role of the AcrAB efflux pump in antibiotic resistance in *A. nosocomialis*

It was recently shown that deletion of the ArpB efflux pump, a homolog of the *A. nosocomialis* AcrAB efflux pump, leads to increased sensitivity to two aminoglycosides, amikacin and tobramycin, in *A. baumannii* 5075 (Tipton *et al.*, 2017). Therefore, we determined the antimicrobial susceptibilities of *A. nosocomialis* wild type and *acrA*- and *acrB*-deletion mutants to a number of antibiotics (Fig. 4 and Table 3). The minimum inhibitory concentrations (MICs) of antimicrobial agents, except for that of acriflavine, were determined using the E-test method. Both *acrA* and *acrB* mutants displayed increased susceptibility to tobramycin and colistin (Table 3), with the *acrB* mutant exhibiting greater sensitivity to colistin than the *acrA* mutant. Susceptibility to acriflavine, determined using the macrodilution method, was greater in both mutants than in the wild type; again, the *acrB* mutant displayed higher susceptibility to acriflavine than the *acrA* mutant (Fig. 4). The MICs of acriflavine for the wild type and mutants were 31.25 and 15.6 μg/ml, respectively. These data emphasize the role of the AcrAB efflux pump in the export of antimicrobial agents and thus in conferring antibiotic resistance.

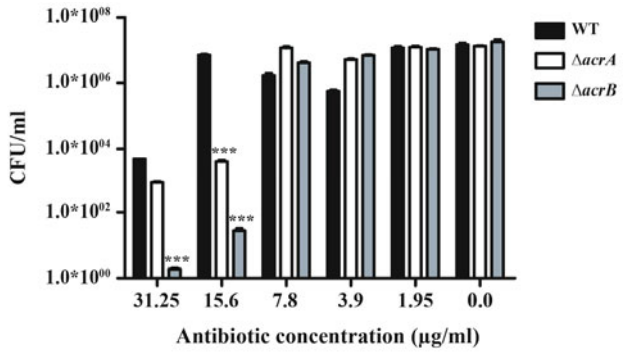

Fig. 4. The AcrAB efflux pump plays a role in antibiotic resistance. Antibiotic resistance of wild type (WT) and *acrA* and *acrB* mutants (Δ*acrA* and Δ*acrB*), measured against acriflavine. The MIC of acriflavine was determined using a macrodilution method. Cultures were treated with different, serially diluted concentrations of acriflavine and incubated for 24 h, after which colony-forming units (CFUs) were counted. Values are means ± SD ($n=3$; *** $P < 0.001$ for mutants vs. wild type). Experiments were conducted on three separate occasions.

Deletion of *acrA* and *acrB* leads to loss of drug efflux

It has been reported that deletion of multidrug efflux pump genes leads to the loss of efflux pump activity (Morita *et al.*, 2001; Li *et al.*, 2003). The efflux activity of wild type and mutant strains was measured by determining the intracellular accumulation of ethidium bromide (Viveiros *et al.*, 2008), which is associated with a time-dependent increase in fluorescence. Active efflux causes reduced accumulation of ethidium bromide and thus a decrease in fluorescence, whereas the loss of efflux pump activity is associated with increased accumulation of ethidium bromide and a higher signal. Upon addition of ethidium bromide, *acrA*- and *acrB*-deletion mutants displayed higher fluorescence signals than the wild type strain, indicating impaired efflux activity in the mutants (Fig. 5).

Table 3. Minimum inhibitory concentrations of antibiotics

Class of antibiotics	Antimicrobial agent	MIC (mg/L)		
		WT[a]	Δ*acrA*[b]	Δ*acrB*[c]
Monobactams	Aztreonam	2	8	8
Cephems	Ceftazidime	0.5	1.5	2
Fluoroquinolones	Ciprofloxacin	0.064	0.125	0.125
Glycylcycline	Tigecyclin	0.125	0.125	0.125
Lipopeptides	Colistin	1	0.5	0.38
Aminoglycosides	Gentamicin	0.19	0.19	0.25
Tetracyclines	Tetracycline	0.75	0.5	0.75
Folate pathway inhibitors	Trimethoprim	8	8	16
Carbapenems	Imipenem	0.094	0.094	0.094
Aminoglycosides	Tobramycin	0.125	0.064	0.094

[a] wild type; [b] *acrA* mutant; [c] *acrB* mutant

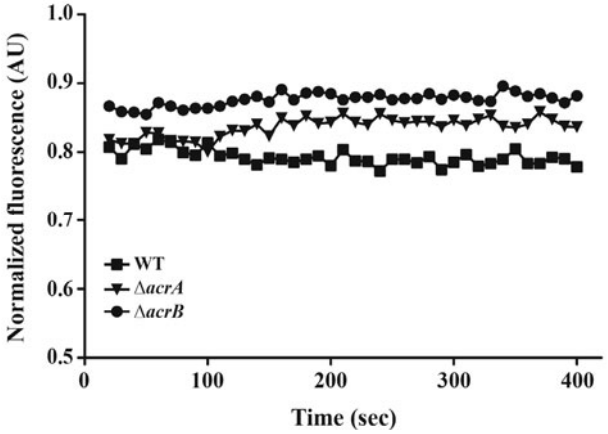

Fig. 5. Ethidium bromide accumulation assay. Strains were cultivated in LB overnight and then diluted to an OD_{600} of 1. Ethidium bromide was added at a final concentration of 50 μM, and changes in fluorescence were measured for 30 min at room temperature. AU, arbitrary unit.

Expression of *acrAB* is downregulated in the *anoR*-deletion mutant

AnoR is a QS regulator that controls the production of the QS signals, N-acyl homoserine lactones (AHLs) (Oh and Choi, 2015), thereby modulating various physiological characteristics such as biofilm formation, motility and virulence, among others, in *A. nosocomialis*. RNA sequencing analyses revealed downregulation of *acrA* (7.3-fold) and *acrB* (5.8-fold) genes in the *anoR*-deletion mutant. These RNA sequencing data were further confirmed by mRNA expression analyses, which revealed that expression of the *acrA* and *acrB* genes was downregulated in the *anoR* mutant, exhibiting 7.7- and 8.7-fold decreases, respectively (Fig. 6). The above results suggest the involvement of the QS regulator, AnoR, in regulating the expression of the multidrug efflux pump genes, *acrA* and *acrB*, in *A. nosocomialis*.

AHL secretion is unaffected in *acrA*- and *acrB*-deletion mutants

It was previously shown that expression of multidrug efflux pumps is important for the secretion of AHLs into the surrounding medium (Evans *et al.*, 1998; Maseda *et al.*, 2004). Therefore, we were curious to determine whether secretion of AHLs is affected by deletion of the AcrAB efflux pump system in *A. nosocomialis*. Bioassays performed to assess the export of AHLs into the surrounding medium showed little difference in AHL secretion in *acrA*- or *acrB*-deletion mutants compared with the wild type (Fig. 7). However, AHL secretion appeared to be reduced in the *acrR* mutant, indicating that AcrR could be an activator of AHL production/secretion. These data demonstrate that secretion of AHLs is not affected by the deletion of *acrAB*, and that AcR could play an important role in the proper functioning of the QS system in *A. nosocomialis*.

AcrR Binds to the promoter region of *anoI* and *anoR*

To determine whether the reduced secretion of AHLs in the *acrR* mutant indicates the direct involvement of AcrR in the transcriptional regulation of *acrAB* genes in *A. nosocomialis*, we carried out an *in silico* analysis to check for the presence of AcrR-binding motifs in the promoter regions of *anoI* (P*anoI*) and *anoR* (P*anoR*) (Fig. 8A). To examine direct interactions between AcrR and P*anoI* or P*anoR*, we performed EMSAs using purified AcrR protein (Subhadra *et al.*, 2018) and 303-bp and 272-bp fragments of the corresponding promoter regions. Upon addition of AcrR, the DNA bands were shifted to higher molecular weights, indicating that AcrR binds to the promoter regions of *anoI* and *anoR* (Fig. 8B). In contrast, no DNA band shift was observed in the case of 16S rDNA, used as a control in place of the promoter fragment, confirming the specificity of promoter-AcrR interactions (Fig. 8B). The AcrR-binding motif, determined based on consensus sequences reported previously in *E. coli* (Su *et al.*, 2007), was located at positions -214 (TTTACAACCATTTGGGCTTAAAAGTGTT) and -217 (TAGAAGCAATATTATGATTGTTGATGTATC) relative to the translational start sites of *anoI* and *anoR*, respectively (Fig. 8A), where the underlined bases represent the consensus residues that match those in the AcrR-binding motif in *E. coli*. These data confirm that AcrR specifically binds to the promoter regions of *anoI* and *anoR*, thus regulating their expression.

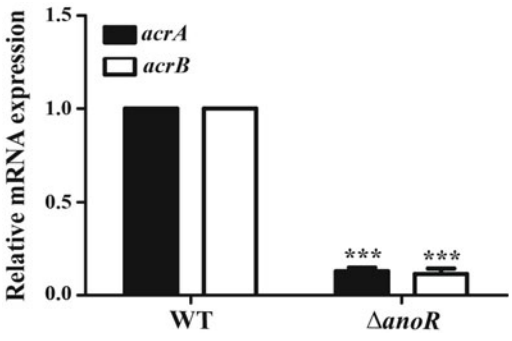

Fig. 6. Downregulation of *acrA* and *acrB* genes in the *anoR*-deletion mutant. Relative expression levels of *acrA* and *acrB* mRNAs in the wild type (WT) and *anoR* mutant (Δ*anoR*) were quantified as described in 'Materials and Methods.' Values are means ± SD ($n = 3$; *** $P < 0.001$ for mutants vs. wild type).

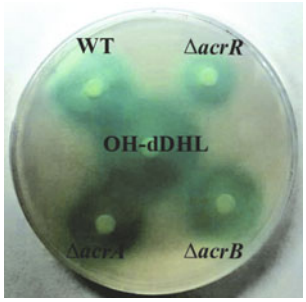

Fig. 7. AHL secretion in deletion mutants. Secretion of AHLs in the wild type (WT) and *acrA*, *acrB*, and *acrR* mutants (Δ*acrA*, Δ*acrB*, and Δ*acrR*) was assessed by bioassay. Strains cultivated overnight were spotted onto chromoplates, and color zone development was monitored after 22 h. A detailed protocol for bioassays is given in 'Materials and Methods.' Bioassays were performed on three separate occasions. OH-dDHL, N-decanoyl-DL-homoserine lactone N-(3-hydroxy-dodecanoyl)-L-homoserine lactone.

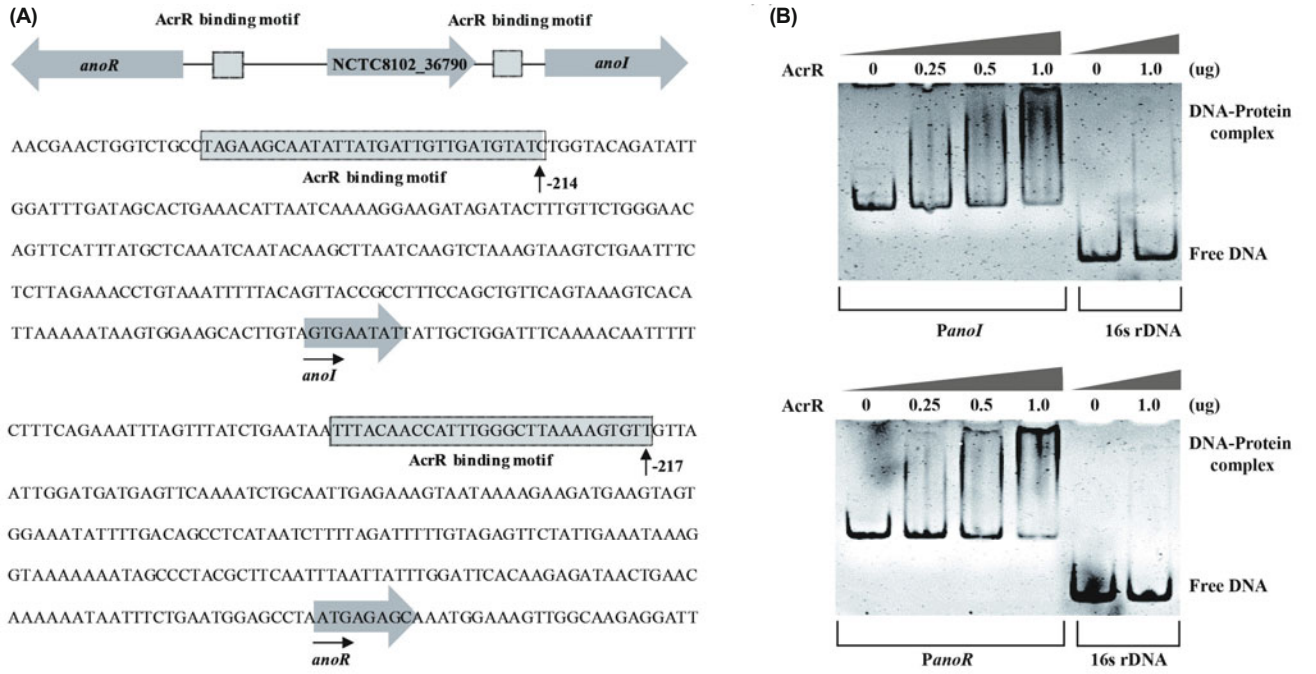

Fig. 8. Validation of the interaction of AcR with the promoter regions of *anoI* and *anoR* by EMSA. (A) Scheme and nucleotide sequences of the promoter regions of *anoI* and *anoR*. Putative AcrR-binding motifs are located in P*anoI* and P*anoR*. The number beneath the indicated AcrR binding site denotes the position of the first nucleotide in the binding motif relative to the translational start site of *anoI* or *anoR*. (B) EMSAs confirmed interactions between AcrR and the promoter regions of *anoI* and *anoR*. The promoter regions of *anoI* and *anoR* were amplified by PCR and treated with different concentrations of purified AcrR. Poly(d[I-C]) (1 μg) was added to each reaction mixture to prevent non-specific binding, and a 16S rDNA sequence was used as a negative control.

Discussion

Multidrug efflux pumps are known to play crucial roles in drug resistance, pathogenicity, biofilm formation, and cell division (Nikaido, 1996; Buckley *et al.*, 2006; Kvist *et al.*, 2008). We recently reported the identification of AcrAB, an RND efflux pump, in *A. nosocomialis* (Subhadra *et al.*, 2018), that shares similarity to the well-studied AcrAB efflux pump in *E. coli*. We also noted that the AcrAB efflux pump is under transcriptional regulation by AcrR in *A. nosocomialis* (Subhadra *et al.*, 2018). In the current study, we characterized the *acrAB* operon encoding AcrA and AcrB, showing that the operon exhibited high similarity to the *arpAB* operon, which plays a role in aminoglycoside resistance in *A. baumannii* 5075 (Tipton *et al.*, 2017). Similar to the case in *E. coli*, both *acrA* and *acrB* in *A. nosocomialis* are located downstream of *acrR* and are divergently transcribed from the same *acrR* promoter.

Deletion of *acrA* and/or *acrB* led to decreased biofilm/pellicle formation in the deletion mutants, indicating the involvement of AcrAB in biofilm/pellicle formation in *A. nosocomialis*. It was previously reported that a number of multidrug efflux systems, including AcrAB-TolC, play an important role in biofilm formation in various Gram-negative bacteria such as *E. coli*, *Klebsiella*, and *S.* Typhimurium (Kvist *et al.*, 2008; Baugh *et al.*, 2012; Schlisselberg *et al.*, 2015). For example, the *E. coli acrB* mutant displays impaired biofilm formation (Kvist *et al.*, 2008; Matsumura *et al.*, 2011), as do *acrAB*- and *acrB*-null mutants of *S.* Typhimurium (Baugh *et al.*, 2012; Schlisselberg *et al.*, 2015). In our study, the ability to form biofilm was not compromised in the *acrA* mutant, a finding in accord with results obtained in *S.* Typhimurium (Baugh *et al.*, 2014). The decreased biofilm/pellicle production in *acrAB* deletion mutants could very well be connected to the role of AcrAB in extracellular matrix formation (Baugh *et al.*, 2014). It has been previously reported that the *csgBA* operon, which synthesizes the curli proteins of the extracellular biofilm matrix of *Salmonella*, is transcriptionally repressed in *acrB* and *tolC* mutants, indicating the involvement of the AcrAB efflux pump in curli production and thereby in biofilm formation. The *csgBA* operon is under the transcriptional repression of the regulator, RamA in *S.* Typhimurium. It has been noticed that *ramA* is overexpressed in the *acrB* and *tolC* mutants, not in *acrA* mutant, suggesting the RamA-mediated repression of curli proteins in *acrB* and *tolC* mutants (Baugh *et al.*, 2014). It is known that there is a significant correlation between multidrug efflux pumps and antibiotic resistance (Magnet *et al.*, 2001; Coyne *et al.*, 2011). For example, deletion of the ArpB efflux pump in *A. baumannii* 5075, a homolog of the *A. nosocomialis* AcrAB efflux pump, increases sensitivity to amikacin and tobramycin. In the current study, *acrA* and *acrB* mutants showed susceptibility to tobramycin, colistin and acriflavine, but, like wild type *A. nosocomialis*, also could not grow in amikacin (data not shown). The AcrAB efflux pump is responsible for exporting a large number of antibiotics, including chloramphenicol, fluoroquinolone, tetracycline, novobiocin, rifampin, fusidic acid, nalidixic acid and β-lactam antibiotics, among

others (Piddock, 2006a). However, the deletion mutants reported here were susceptible only to tobramycin, colistin, and acriflavine. The lack of susceptibility of deletion mutants to the majority of antibiotics tested indicates that other multidrug efflux pumps are likely used for the export of these antibiotics in cases where AcrAB is non-functional (Schlisselberg *et al.*, 2015). These data suggest that administration of specific antibiotics together with efflux pump inhibitors might improve therapeutic strategies for controlling *A. nosocomialis* infections.

It has been reported that multidrug efflux pumps and QS systems involved in cell-cell communication are interrelated in many bacteria (Pearson *et al.*, 1999; Maseda *et al.*, 2004; Chan *et al.*, 2007). The role of multidrug efflux pumps in QS was first described in *P. aeruginosa*, where the MexAB-OprM efflux pump transports hydrophobic AHLs across the cell membrane (Pearson *et al.*, 1999). Overexpression of MexAB-OprM results in increased secretion of AHLs, which, in turn, leads to decreased expression of virulence factors controlled by QS (Evans *et al.*, 1998). In addition, the lack of MexAB-OprM activity contributes to intracellular accumulation of AHL, which adversely affects QS (Evans *et al.*, 1998). Deletion of the *acrAB* operon in *A. nosocomialis* led to the loss of AcrAB efflux pump activity and could lead to the intracellular accumulation of AHLs. However, bioassays revealed no difference in the secretion of AHLs between the deletion mutants and the wild type. Thus, it is reasonable to speculate that AHLs are secreted via other efflux pumps in the mutants in the absence of functional AcrAB. RNA sequencing data and mRNA expression analyses further showed that deletion of *anoR* leads to decreased expression of the *acrAB* operon in *A. nosocomialis*. These data are in accord with findings from *P. aeruginosa,* in which the expression of *mexAB-oprM* is limited by the intracellular concentration of AHLs (Maseda *et al.*, 2004). Likewise, overexpression of the QS regulator, SdiA, in *E. coli* contributes to increased AcrA and AcrB protein levels (Rahmati *et al.*, 2002). Thus, decreased expression of the AcrAB efflux pump in the *anoR* mutant could very well be attributable to low intracellular AHLs levels, given that AnoR is important for optimal production of autoinducers.

Bioassays revealed that AHL secretion is limited in the *acrR*-deletion mutant of *A. nosocomialis* (Fig. 7). *In silico* analyses revealed that AcrR specifically binds to the promoter regions of *anoI* and *anoR*, controlling their expression, a finding that was further confirmed by EMSAs. These data indicate that AcrR might be an activator of *anoI* and *anoR* expression in *A. nosocomialis*. However, further studies are required to confirm the transcriptional regulation of *anoI* and *anoR* by AcrR. It was previously reported that AcrR controls transcription of the global regulatory genes, *marRAB* and *soxRS*, in *E. coli* (Lee *et al.*, 2014). This suggests the possibility that AcrR sits at a higher level in the regulatory network and therefore might control different regulons, including this aspect of the QS system. It is also known that the AcrAB efflux pump is under the dual regulation of AcrR and OmpR in *Yersinia enterocolitica* (Raczkowska *et al.*, 2015). OmpR acts as a transcriptional activator by binding to the promoter region of *acrAB,* where one of its binding motifs overlaps with the AcrR-binding motif. Thus, we concluded that OmpR induces the expression of *acrAB* operon in two ways: by directly binding to P*acrAB,* and by preventing binding of the repressor, AcrR (Raczkowska *et al.*, 2015). In the current study, *in silico* analyses and EMSAs showed that the AcrAB efflux pump is transcriptionally regulated by OmpR (Supplementary data Fig. S2). OmpR binds to multiple binding motifs in P*acrAB*, one of which overlaps with the AcrR-binding motif in *A. nosocomialis,* as is the case in *Y. enterocolitica*. This indicates that OmpR might directly control the expression of AcrAB by binding to P*acrAB* as well as by inhibiting the repressive effect of AcrR in *A. nosocomialis*. It was previously established that, in addition to AcrR, four other regulators–MarA, SoxS, Rob, and RamA–also regulate *acrAB* expression in response to various inducers, such as indole, bile salts and paraquat, among others, in other bacterial strains (Ma *et al.*, 1996; Randall and Woodward, 2002; Rosenberg *et al.*, 2003; Schneiders *et al.*, 2003). However, the factors that induce OmpR-mediated expression of *acrAB* are not yet known.

In summary, we report the functional characterization of the multidrug efflux pump, AcrAB, in *A. nosocomialis*. Though we could not include the complemented mutants for *acrA* and *acrB* in the current study, experimental data obtained using wild type and mutant strains showed the functional roles of *acrA* and *acrB* in *A. nosocomialis* regarding the antimicrobial susceptibility and biofilm/pellicle formation. The data obtained in this study are in accordance with the previous reports in *A. baumannii* and other gram-negative bacterial species (Kvist *et al.*, 2008; Baugh *et al.*, 2012; Schlisselberg *et al.*, 2015; Tipton *et al.*, 2017).

We found that the AcrAB efflux pump is under transcriptional regulation by AnoR in addition to AcrR, and plays an important role in antibiotic resistance and biofilm/pellicle formation in *A. nosocomialis*. We further found evidence that AcrR might regulate expression of the QS genes, *anoI* and *anoR*, in *A. nosocomialis*. However, further studies are needed for an in-depth understanding of the transcriptional regulation of AcrAB in *A. nosocomialis* under various environmental conditions and the interrelationship of this efflux pump with the QS system. Knowledge gained from such studies could be utilized for developing efflux pump inhibitors, which are invaluable tools for controlling multidrug-resistant nosocomial pathogens.

Acknowledgments

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Conflict of Interest

The authors confirm that there are no conflicts of interest.

References

Alves, C. and Cunha, C. 2012. Electrophoretic mobility shift assay: analyzing protein - Nucleic acid interactions, pp. 205–229. Gel Electrophoresis-Advanced Techniques. INTECH, Rijeka, Croatia.

Baugh, S., Ekanayaka, A.S., Piddock, L.J., and Webber, M.A. 2012. Loss of or inhibition of all multidrug resistance efflux pumps of *Salmonella enterica* serovar Typhimurium results in impaired ability to form a biofilm. *J. Antimicrob. Chemother.* **67**, 2409–2417.

Baugh, S., Phillips, C.R., Ekanayaka, A.S., Piddock, L.J., and Webber, M.A. 2014. Inhibition of multidrug efflux as a strategy to prevent biofilm formation. *J. Antimicrob. Chemother.* **69**, 673–681.

Buckley, A.M., Webber, M.A., Cooles, S., Randall, L.P., La Ragione, R.M., Woodward, M.J., and Piddock, L.J. 2006. The AcrAB-TolC efflux system of *Salmonella enterica* serovar Typhimurium plays a role in pathogenesis. *Cell. Microbiol.* **8**, 847–856.

Bunikis, I., Denker, K., Östberg, Y., Andersen, C., Benz, R., and Bergstrom, S. 2008. An RND-type efflux system in *Borrelia burgdorferi* is involved in virulence and resistance to antimicrobial compounds. *PLoS Pathog.* **4**, e1000009.

Chan, Y.Y., Bian, H.S., Tan, T.M., Mattmann, M.E., Geske, G.D., Igarashi, J., Hatano, T., Suga, H., Blackwell, H.E., and Chua, K.L. 2007. Control of quorum sensing by a *Burkholderia pseudomallei* multidrug efflux pump. *J. Bacteriol.* **189**, 4320–4324.

CLSI (Clinical and Laboratory Standards Institute). 2017. Performance standards for antimicrobial susceptibility testing. 27th edn. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania, USA.

CLSI (Clinical and Laboratory Standards Institute). 2018. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania, USA.

Coyne, S., Courvalin, P., and Périchon, B. 2011. Efflux-mediated antibiotic resistance in *Acinetobacter* spp. *Antimicrob. Agents Chemother.* **55**, 947–953.

Coyne, S., Rosenfeld, N., Lambert, T., Courvalin, P., and Périchon, B. 2010. Overexpression of resistance-nodulation-cell division pump AdeFGH confers multidrug resistance in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* **54**, 4389–4393.

Damier-Piolle, L., Magnet, S., Brémont, S., Lambert, T., and Courvalin, P. 2008. AdeIJK, a resistance-nodulation-cell division pump effluxing multiple antibiotics in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* **52**, 557–562.

Evans, K., Passador, L., Srikumar, R., Tsang, E., Nezezon, J., and Poole, K. 1998. Influence of the MexAB-OprM multidrug efflux system on quorum sensing in *Pseudomonas aeruginosa*. *J. Bacteriol.* **180**, 5443–5447.

Giles, S.K., Stroeher, U.H., Eijkelkamp, B.A., and Brown, M.H. 2015. Identification of genes essential for pellicle formation in *Acinetobacter baumannii*. *BMC Microbiol.* **15**, 116.

Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166**, 557–580.

Hirakata, Y., Srikumar, R., Poole, K., Gotoh, N., Suematsu, T., Kohno, S., Kamihira, S., Hancock, R.E., and Speert, D.P. 2002. Multidrug efflux systems play an important role in the invasiveness of *Pseudomonas aeruginosa*. *J. Exp. Med.* **196**, 109–118.

Koronakis, V., Eswaran, J., and Hughes, C. 2004. Structure and function of TolC: the bacterial exit duct for proteins and drugs. *Annu. Rev. Biochem.* **73**, 467–489.

Kvist, M., Hancock, V., and Klemm, P. 2008. Inactivation of efflux pumps abolishes bacterial biofilm formation. *Appl. Environ. Microbiol.* **74**, 7376–7382.

Lee, J.O., Cho, K.S., and Kim, O.B. 2014. Overproduction of AcrR increases organic solvent tolerance mediated by modulation of SoxS regulon in *Escherichia coli*. *Appl. Microbiol. Biotechnol.* **98**, 8763–8773.

Lee, H.W., Koh, Y.M., Kim, J., Lee, J.C., Lee, Y.C., Seol, S.Y., Cho, D.T., and Kim, J. 2008. Capacity of multidrug-resistant clinical isolates of *Acinetobacter baumannii* to form biofilm and adhere to epithelial cell surfaces. *Clin. Microbiol. Infect.* **14**, 49–54.

Li, Y., Mima, T., Komori, Y., Morita, Y., Kuroda, T., Mizushima, T., and Tsuchiya, T. 2003. A new member of the tripartite multidrug efflux pumps, MexVW-OprM, in *Pseudomonas aeruginosa*. *J. Antimicrob. Chemother.* **52**, 572–575.

Li, X.Z. and Nikaido, H. 2004. Efflux-mediated drug resistance in bacteria. *Drugs* **64**, 159–204.

Livak, K.J. and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**, 402–408.

Lynch, S.V., Dixon, L., Benoit, M.R., Brodie, E.L., Keyhan, M., Hu, P., Ackerley, D.F., Andersen, G.L., and Matin, A. 2007. Role of the *rapA* gene in controlling antibiotic resistance of *Escherichia coli* biofilms. *Antimicrob. Agents Chemother.* **51**, 3650–3658.

Ma, D., Alberti, M., Lynch, C., Nikaido, H., and Hearst, J.E. 1996. The local repressor AcrR plays a modulating role in the regulation of *acrAB* genes of *Escherichia coli* by global stress signals. *Mol. Microbiol.* **19**, 101–112.

Ma, D., Cook, D.N., Alberti, M., Pon, N.G., Nikaido, H., and Hearst, J.E. 1993. Molecular cloning and characterization of *acrA* and *acrE* genes of *Escherichia coli*. *J. Bacteriol.* **175**, 6299–6313.

Ma, D., Cook, D.N., Alberti, M., Pon, N.G., Nikaido, H., and Hearst, J.E. 1995. Genes *acrA* and *acrB* encode a stress-induced efflux system of *Escherichia coli*. *Mol. Microbiol.* **16**, 45–55.

Magnet, S., Courvalin, P., and Lambert, T. 2001. Resistance-nodulation-cell division-type efflux pump involved in aminoglycoside resistance in *Acinetobacter baumannii* strain BM4454. *Antimicrob. Agents Chemother.* **45**, 3375–3380.

Maseda, H., Sawada, I., Saito, K., Uchiyama, H., Nakae, T., and Nomura, N. 2004. Enhancement of the *mexAB-oprM* efflux pump expression by a quorum-sensing autoinducer and its cancellation by a regulator, MexT, of the *mexEF-oprN* efflux pump operon in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **48**, 1320–1328.

Matsumura, K., Furukawa, S., Ogihara, H., and Morinaga, Y. 2011. Roles of multidrug efflux pumps on the biofilm formation of *Escherichia coli* K-12. *Biocontrol Sci.* **16**, 69–72.

Morita, Y., Komori, Y., Mima, T., Kuroda, T., Mizushima, T., and Tsuchiya, T. 2001. Construction of a series of mutants lacking all of the four major *mex* operons for multidrug efflux pumps or possessing each one of the operons from *Pseudomonas aeruginosa* PAO1: MexCD-OprJ is an inducible pump. *FEMS Microbiol. Lett.* **202**, 139–143.

Nikaido, H. 1996. Multidrug efflux pumps of Gram-negative bacteria. *J. Bacteriol.* **178**, 5853–5859.

Oh, M.H. and Choi, C.H. 2015. Role of LuxIR homologue AnoIR in *Acinetobacter nosocomialis* and the effect of virstatin on the expression of *anoR* gene. *J. Microbiol. Biotechnol.* **25**, 1390–1400.

Oh, M.H., Lee, J.C., Kim, J., Choi, C.H., and Han, K. 2015. Simple method for markerless gene deletion in multidrug-resistant *Acinetobacter baumannii*. *Appl. Environ. Microbiol.* **81**, 3357–3368.

Park, S.Y., Lee, S.J., Oh, T.K., Oh, J.W., Koo, B.T., Yum, D.Y., and Lee, J.K. 2003. AhlD, an *N*-acyl homoserine lactonase in *Arthrobacter* sp., and predicted homologues in other bacteria. *Microbiology* **149**, 1541–1550.

Pearson, J.P., Van Delden, C., and Iglewski, B.H. 1999. Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. *J. Bacteriol.* **181**, 1203–1210.

Piddock, L.J. 2006a. Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. *Clin. Microbiol. Rev.* **19**, 382–402.

Piddock, L.J. 2006b. Multidrug-resistance efflux pumps - not just for resistance. *Nat. Rev. Microbiol.* **4**, 629–636.

Poole, K., Krebes, K., McNally, C., and Neshat, S. 1993. Multiple

antibiotic resistance in *Pseudomonas aeruginosa*: Evidence for involvement of an efflux operon. *J. Bacteriol.* **175**, 7363–7372.

Pumbwe, L., Randall, L.P., Woodward, M.J., and Piddock, L.J. 2004. Expression of the efflux pump genes *cmeB*, *cmeF* and the porin gene *porA* in multiple-antibiotic-resistant *Campylobacter jejuni*. *J. Antimicrob. Chemother.* **54**, 341–347.

Raczkowska, A., Trzos, J., Lewandowska, O., Nieckarz, M., and Brzostek, K. 2015. Expression of the AcrAB components of the AcrAB-TolC multidrug efflux pump of *Yersinia enterocolitica* is subject to dual regulation by OmpR. *PLoS One* **10**, e0124248.

Rahmati, S., Yang, S., Davidson, A.L., and Zechiedrich, E.L. 2002. Control of the AcrAB multidrug efflux pump by quorum-sensing regulator SdiA. *Mol. Microbiol.* **43**, 677–685.

Randall, L.P. and Woodward, M.J. 2002. The multiple antibiotic resistance (*mar*) locus and its significance. *Res. Vet. Sci.* **72**, 87–93.

Rosenberg, E.Y., Bertenthal, D., Nilles, M.L., Bertrand, K.P., and Nikaido, H. 2003. Bile salts and fatty acids induce the expression of *Escherichia coli* AcrAB multidrug efflux pump through their interaction with Rob regulatory protein. *Mol. Microbiol.* **48**, 1609–1619.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. Molecular cloning: a laboratory manual. 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA.

Schlisselberg, D.B., Kler, E., Kisluk, G., Shachar, D., and Yaron, S. 2015. Biofilm formation ability of *Salmonella enterica* serovar Typhimurium *acrAB* mutants. *Int. J. Antimicrob. Agents* **46**, 456–459.

Schneiders, T., Amyes, S.G., and Levy, S.B. 2003. Role of AcrR and RamA in fluoroquinolone resistance in clinical *Klebsiella pneumoniae* isolates from Singapore. *Antimicrob. Agents Chemother.* **47**, 2831–2837.

Simon, R., Priefer, U., and Pühler, A. 1983. A broad host range mobilization system for *in vivo* genetic engineering transposon mutagenesis in Gram negative bacteria. *Nat. Biotechnol.* **1**, 784–791.

Studier, F.W. and Moffatt, B.A. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **189**, 113–130.

Su, C.C., Rutherford, D.J., and Yu, E.W. 2007. Characterization of the multidrug efflux regulator AcrR from *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **361**, 85–90.

Subhadra, B., Kim, J., Kim, D.H., Woo, K., Oh, M.H., and Choi, C.H. 2018. Local repressor AcrR regulates AcrAB efflux pump required for biofilm formation and virulence in *Acinetobacter nosocomialis*. *Front. Cell. Infect. Microbiol.* **8**, 270.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* **30**, 2725–2729.

Tipton, K.A., Farokhyfar, M., and Rather, P.N. 2017. Multiple roles for a novel RND-type efflux system in *Acinetobacter baumannii* AB5075. *Microbiologyopen* **6**, e00418.

Viveiros, M., Martins, A., Paixão, L., Rodrigues, L., Martins, M., Couto, I., Fähnrich, E., Kern, W.V., and Amaral, L. 2008. Demonstration of intrinsic efflux activity of *Escherichia coli* K-12 AG100 by an automated ethidium bromide method. *Int. J. Antimicrob. Agents* **31**, 458–462.

Zhang, L., Murphy, P.J., Kerr, A., and Tate, M.E. 1993. *Agrobacterium* conjugation and gene regulation by *N*-acyl-L-homoserine lactones. *Nature* **362**, 446–448.