Local Repressor AcrR Regulates AcrAB Efflux Pump Required for Biofilm Formation and Virulence in Acinetobacter nosocomialis

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Multidrug efflux systems contribute to antimicrobial resistance and pathogenicity in bacteria. Here, we report the identification and characterization of a transcriptional regulator AcrR controlling the yet uncharacterized multidrug efflux pump, AcrAB in Acinetobacter nosocomialis. In silico analysis revealed that the homologs of AcrR and AcrAB are reported in the genomes of many other bacterial species. We confirmed that the genes encoding the AcrAB efflux pump, acrA and acrB forms a polycistronic operon which is under the control of acrR gene upstream of acrA. Bioinformatic analysis indicated the presence of AcrR binding motif in the promoter region of acrAB operon and the specific binding of AcrR was confirmed by electrophoretic mobility shift assay (EMSA). The EMSA data showed that AcrR binds to −89 bp upstream of the start codon of acrA. The mRNA expression analysis depicted that the expression of acrA and acrB genes are elevated in the deletion mutant compared to that in the wild type confirming that AcrR acts as a repressor of acrAB operon in A. nosocomialis. The deletion of acrR resulted in increased motility, biofilm/pellicle formation and invasion in A. nosocomialis. We further analyzed the role of AcrR in A. nosocomialis pathogenesis in vivo using murine model and it was shown that acrR mutant is highly virulent inducing severe infection in mouse leading to host death. In addition, the intracellular survival rate of acrR mutant was higher compared to that of wild type. Our data demonstrates that AcrR functions as an important regulator of AcrAB efflux pump and is associated with several phenotypes such as motility, biofilm/pellicle formation and pathogenesis in A. nosocomialis.

Keywords: A. nosocomialis, efflux pump, AcrR, antibiotic resistance, quorum sensing, biofilm, virulence

INTRODUCTION

Acinetobacter spp. are important opportunistic bacterial pathogens primarily associated with nosocomial infections worldwide. These are the causative organisms for a number of pathological conditions including bacteremia, pneumonia, meningitis, urinary tract infections, and wound infections (Peleg et al., 2008; Lee et al., 2011; Nemec et al., 2011). The medically relevant Acinetobacter spp. predominantly come from a single group, designated as Acinetobacter baumannii (Ab) group which includes A. baumannii, Acinetobacter nosocomialis, Acinetobacter pittii, Acinetobacter seifertii, and Acinetobacter dijkshoorniae. All the members of the Ab group...
are opportunistic pathogens and have the capacity to acquire multidrug resistance phenotype (Nemec et al., 2015; Cosgaya et al., 2016; Mari-Almirall et al., 2017).

A. nosocomialis is the most comprehensively studied strain in the Ab group second to A. baumannii. A. baumannii is reported to rapidly develop resistance to multiple antibiotics and is one of the most challenging pathogens for healthcare institutions (Fournier and Richet, 2006; Antunes et al., 2014; Doi et al., 2015).

The increasing rate of multidrug resistance (MDR) phenotypes associated with Acinetobacter infections are so alarming that the Centers for Disease Control and Prevention (CDC) has listed Acinetobacter as a severe threat to mankind and last line therapeutic options like carbapenems, tigecycline, and colistin are now recommended to treat majority of Acinetobacter infections (Peleg and Hooper, 2010; CDC, 2013). Moreover, according to the recent epidemiological data, MDR strains acquire multidrug resistance phenotype faster than any of the other Gram-negative bacteria reported over the last decade (Giammanco et al., 2017).

Multidrug efflux pumps contribute to antibiotic resistance by extruding out drugs and are found in almost all bacterial species. These pumps have been categorized into five families based on the basis of sequence similarity: resistance-nodulation-cell division (RND), the major facilitator, small multidrug resistance, multidrug and toxic compound extrusion, and ATP-binding cassette (ABC) families (Putman et al., 2000; Paulsen et al., 2001). In Gram negative bacteria, RND family efflux pumps are mostly effective in resistance to antibiotics (Nikaido, 1996; Murakami et al., 2002, 2006) and consist of an RND protein (the inner membrane component), membrane fusion protein (MFP: the periplasmic component), and the outer membrane protein (OMP: the outer membrane protein). The RND pumps were first reported in Escherichia coli and Pseudomonas aeruginosa independently and those include AcrAB-ToIC (E. coli) (Ma et al., 1993, 1995), MexAB-OprM, MexCD-OprJ, MexEF-OprN etc. (P. aeruginosa) (Poole et al., 1993, 1996; Gotoh et al., 1995; Köhler et al., 1997). In E. coli, the increased level of antibiotic resistance is attributed majorly by this housekeeping efflux system AcrAB-ToIC (Thanassi et al., 1997). The substrate profile of the AcrAB-ToIC efflux pump in E. coli includes chloramphenicol, fluoroquinolone, tetracycline, novobiocin, rifampin, fusidic acid, nalidixic acid, and ß-lactam antibiotics (Piddock, 2006). Apart from E. coli, the function of AcrAB efflux pump or its homologs on multidrug resistance has been reported in a number of bacterial strains including pseudomonads, Salmonella enterica serovar Typhimurium, Enterobacter aerogenes, Klebsiella pneumoniae, Enterobacter cloacae, Campylobacter spp., and Borrelia burgdorferi, etc. (Poole et al., 1993; Pumbwe et al., 2004; Buckley et al., 2006; Bunikis et al., 2008). Like that in E. coli, the AcrAB-ToIC system in S. enterica is reported to actively expel different classes of antimicrobial agents such as quinolones, chloramphenicol, tetracycline, and nalidixic acid (Poole, 2007; Nishino et al., 2009).

The efflux pumps are almost exclusive to A. baumannii, A. pittii, and A. nosocomialis and their orthologues are distributed in other bacterial pathogens, such as Pseudomonas spp. The AdeABC efflux pump of A. baumannii confers resistance to various classes of antibiotics including aminoglycosides, tetracyclines, erythromycin, chloramphenicol, trimethoprim, fluoroquinolones, some ß-lactams, tigecycline etc. (Magnet et al., 2001; Marchand et al., 2004).

The adeABC operon is found in 80% of the clinical isolates and its expression is tightly regulated by the two-component system, AdeRS (Marchand et al., 2004). A second RND efflux pump was reported in A. baumannii known as the AdeJK which contributes resistance to ß-lactams, chloramphenicol, tetracycline, tigecycline, erythromycin, lincosamides, fluoroquinolones, fusidic acid, novobiocin, rifampin, trimethoprim, acridine, safranin, pyrone, and sodium dodecyl sulfate (Laurence et al., 2007). The RND efflux pump, AdeFGH, encoded by adeFGH operon provides high-level resistance to fluoroquinolones, chloramphenicol, trimethoprim, clindamycin etc. and is under the control of a putative LysR-type transcriptional regulator, named adel, which is located upstream of the adeFGH operon (Coyne et al., 2010a,b). Besides their role in antibiotic resistance, RND efflux pumps contribute to virulence and biofilm formation in A. baumannii (Richmond et al., 2016).

AroN is a LuxR-type transcriptional regulator controlling the production of quorum sensing signals, N-acyl homoserine lactones (AHLS) in A. nosocomialis ATCC 17903 (Oh and Choi, 2015). Recently, we analyzed the whole transcriptome of A. nosocomialis wild-type (WT) and the anoR deletion mutant (unpublished data), and it revealed the downregulation of two uncharacterized efflux pump genes, NCTC8102_00280 and NCTC8102_00290. In silico analysis pointed out that these genes belong to the RND-type transport system and many homologs are found in the bacterial community. The NCTC8102_00280 and NCTC8102_00290 showed identity to the acrA and acrB genes of the acrAB operon, constituting the AcrAB-ToIC efflux pump (Okusu et al., 1996) of E. coli. The regulation of expression of RND pumps are tightly regulated by an intricate balance between local regulators (generally repressors) and global regulators. It has been reported previously that acrAB is under the control of a local repressor, AcrR in E. coli (Ma et al., 1996). Further in silico analysis of the A. nosocomialis genome revealed an AcrR homolog upstream of acrA of the acrAB operon. In this study, we report the regulation of acrAB efflux pump by AcrR and the data indicate that AcrR plays a major role in motility, biofilm/pellicle formation and pathogenesis in A. nosocomialis by repressing the acrAB operon.

**MATERIALS AND METHODS**

**Bacterial Strains, Plasmids, and Culture Conditions**

Bacterial strains and plasmids used in this study are shown in Table 1. Unless otherwise stated, E. coli strains were grown 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 or 37°C in either LB or Mueller Hinton (MH) media (Difco). When appropriate, antibiotics were added to media for E. coli (kanamycin, 50 µg/ml; chloramphenicol,
20 µg/ml as well as A. nosocomialis (kanamycin, 30 µg/ml; ampicillin, 100 µg/ml). Human avelar epithelial cell line (A549) was cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, WELGENE Inc., Korea) supplemented with 10% fetal bovine serum (FBS, WELGENE Inc., Korea) and penicillin G (100 U/ml)/streptomycin (100 µg/ml). The cells were maintained at 37°C in 5% (vol/vol) CO2 in 75-cm² tissue culture flasks until used.

DNA Manipulations

Standard cloning procedures were followed in this study (Sambrook et al., 1989). Oligonucleotides were purchased from Macrogen Co., Ltd., Korea and all the oligonucleotides used in this study are given in Table 2. The genomic DNA from A. nosocomialis and the plasmid DNA from E. coli were isolated using the PureHelix™ Genomic DNA Prep Kit (Nanohelix Co. Ltd., Korea) and AccuPrep™ Plasmid Extraction Kit (Bioneer Corp., Korea), respectively. The plasmids were introduced into E. coli and A. nosocomialis by heat shock method (Hanahan, 1983) and conjugation (Oh et al., 2015), respectively. PrimerSTAR GXL Taq DNA polymerase (TaKaRa, Japan) was used for polymerase chain reaction (PCR) amplification of DNA fragments. The DNA fragments were purified or eluted from gel using HiGene™ Gel & PCR Purification System (Biofact Co., Ltd., Korea). Restriction and DNA modifying enzymes were purchased from New England Biolabs, USA. The nucleotide sequence of all recombiant strains and mutants were confirmed by sequencing (Macrogen Co., Ltd., Korea).

In Silico Analysis

In silico analysis was performed to identify homologs of acrAB operon and acrR, using the A. nosocomialis ATCC 17903 (NCTC 8102) genome sequence retrieved from GenBank (GenBank accession no. CP029351). Phylogenetic analysis and molecular evolutionary analyses were conducted to determine the relationship of acrR of A. nosocomialis with that of other related strains. The strains used for the construction of phylogenetic tree are given in Supplementary Table 1. Phylogenetic tree was constructed using MEGA 6.06 (Tamura et al., 2013). The sequences were aligned using MUSCLE (Edgar, 2004) and pairwise distances were estimated using the Maximum Likelihood approach. In order to find out the binding motif of AcrR in the promoter region of acrA/acrR (PacrA/acrR) of A. nosocomialis, multiple sequence alignment was carried out with the PacrA/acrR and the known AcrR binding motif from E. coli (Su et al., 2007).

Overexpression and Purification of His6-AcrR

For the overexpression of hexahistidyl-tagged AcrR (His6-AcrR), the complete coding region of acrR (624 bp) of A. nosocomialis was amplified by PCR and subcloned into His-tag expression vector, pET28a yielding pET-acrR. The recombinant plasmid was introduced into E. coli strain BL21(DE3) by transformation. The AcrR was overexpressed by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at an OD600 of 0.5 and incubated the culture further for 5 h at 30°C. Induced cells were harvested by centrifugation and lysed by sonication (Vibratome, Sonics & Materials, Inc., USA) in lysis buffer [20 mM Tris-HCl (pH 8), 500 mM NaCl and 5 mM imidazole]. After centrifugation, the soluble supernatant was mixed with nickel-nitrotriacetic acid–agarose solution (Qiagen, Germany) and loaded into polypropylene column (Thermo Fisher Scientific, USA). The column was washed with lysis buffer and the protein was eluted in elution buffer with varying imidazole concentration [20 mM Tris-HCl (pH 8), 500 mM NaCl, and 150/300 mM imidazole]. The purified AcrR was dialyzed overnight against phosphate buffered saline (PBS) to remove imidazole and directly used for the promoter binding assay.

Electrophoretic Mobility Shift Assay (EMSA)

EMSA was carried out to check the binding of AcrR to the promoter region of acrA/acrR according to the protocol described earlier (Alves and Cunha, 2012). A promoter fragment of 148 bp and 16s rDNA of 149 was amplified by PCR using the oligonucleotide primers listed in Table 2. In the binding assay, 200 ng of the PacrA/acrR fragment was incubated with varying amounts of purified His6-AcrR (0 to 0.5 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)]]]. To confirm the binding specificity of AcrR, 16s rDNA was included in the EMSA reaction mixture instead of the promoter fragment. The binding assay was carried out at room temperature (RT) for 30 min and the reaction mixtures were electrophoresed in 6% prerunned (20 min at 80 V) native polyacrylamide gel in 1x Tris/Borate/EDTA (TBE) buffer for 1 h at 80 V followed by staining with ECOred (MOSAICON, Korea) and visualization.

RNA Extraction and Real-Time Quantitative Reverse Transcription PCR (RT-PCR)

Cultures were inoculated into LB medium and incubated until the exponential phase. Total RNA was extracted with High Pure RNA Isolation Kit (Roche Diagnostics GmbH, Germany) according to the manufacturer’s instructions. Complementary DNA (cDNA) was synthesized from 1 µg of DNase-treated total RNA with Reverse Transcription Master Premix (ELPIS Biotech. Inc., Korea) per manufacturer’s instructions. Quantitative RT-PCR was performed in triplicates using StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) with Power SYBR® Green PCR Master Mix (Applied Biosystems, USA) and indicated primers (Table 2). Gene expression was normalized by the ΔΔCT method (Livak and Schmittgen, 2001) and 16s rRNA was used as a reference.

Construction of acrR Deletion Mutant and Complemented Strain

To construct the deletion mutant of acrR, markerless gene deletion method was followed as described previously (Oh et al., 2015). The complete open reading frame of the acrR (624 bp) was deleted by overlap extension PCR. For this, the upstream
TABLE 1 | Bacterial strains and plasmids used in this study.

| Strain/Plasmid | Relevant characteristics | References |
|----------------|--------------------------|------------|
| **Acinetobacter nosocomialis STRAINS** | | |
| ATCC 17903 | Type strain | ATCC |
| ΔacrR | acrR deletion mutant of ATCC 17903 | This study |
| **Escherichia coli STRAINS** | | |
| DH5α | F- thi-1 end A1 hsdR17 (r− m−) supE44 ΔlacU169 (φ80lacZΔM15) recA1 gyrA96 relA | Hanahan, 1983 |
| DH5α λ, pir | supE44 ΔlacU169(φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA λpir (phage lysogen); plasmid replication | Laboratory collection |
| S17-1 λ, pir | λpir lysogen; thi pro hsdR hsdM<sup>+</sup> recA RP4-2 | Simon et al., 1983 |
| BL21(DE3) | ompT hsdS<sup>B</sup>B<sup>12</sup> m<sup>B</sup> gal dcm (DE3) | Studier and Moffatt, 1986 |
| **PLASMIDS** | | |
| pUC4K | pUC4 with nptI; Ap<sup>r</sup>; Km<sup>f</sup> | Pharmacia |
| pHKD01 | pDS132, multicloning sites; ori<sup>F</sup>R6K sacB; Cm<sup>f</sup> | Oh et al., 2015 |
| pOH959 | pHKD01 with ΔacrR:nptI; Cm<sup>f</sup>, Km<sup>f</sup> | This study |
| pOH993 | pHKD01 with the coding region of acrR under control of its native promoter with nptI; Cm<sup>f</sup>, Km<sup>f</sup> | This study |
| pET-acrR | pET_28a carrying the coding region of acrR | This study |

*<sup>NP</sup>*, trimetoprim resistant; <sup>S</sup>r, streptomycin resistant; <sup>A</sup>p, ampicillin resistant; <sup>K</sup>m, kanamycin resistant; <sup>C</sup>m, chloramphenicol resistant.

and downstream regions of the acrR were amplified using two sets of primer pair (AcrR01F and AcrR01R; AcrR02F and AcrR02R). nptI conferring kanamycin resistance was amplified from pUC4K using the primers, U1 and U2. Overlap extension PCR was carried out to anneal the three PCR products at their overlapping regions using the primers AcrR01F and U2. The fusion product was ligated into FspI-digested pHKD01 to generate pOH993. The recombinant plasmid, pOH993 was introduced into ΔacrR deletion mutant by conjugation and the recombinants were screened as described previously (Oh et al., 2015).

**Motility Test**

Surface-associated motility was examined on MH plate containing 0.25% Eiken soft agar (Eiken chemical, Co., Ltd., Japan) as described previously (Skiebe et al., 2012). In order to minimize the variation between plates, 30 ml of the motility agar was poured to each plate in the laminar flow hood and the plates were dried for 30 min. *A. nosocomialis* WT, acrR deletion mutant and complemented mutant strains cultivated in MH broth overnight were diluted to an OD<sub>600nm</sub> of 1 and 5 μl of the samples were spotted onto the motility plate. The plates were incubated at 30°C for 18 h and the motility on top of agar was observed. The plates were prepared fresh for each surface motility experiment and the test was repeated on at least three separate occasions.

**Biofilm/Pellicle Formation Assay**

Biofilm and pellicle formation assays were adapted from previously described protocols with slight modifications (Lee et al., 2008; Giles et al., 2015). For biofilm and pellicle formation assay, *A. nosocomialis* WT, acrR deletion mutant and complemented strains were cultivated in MH broth at 37°C. The overnight cultures were diluted to an OD<sub>600nm</sub> of 1 and 25 μl of the diluted cultures were added to 5 ml of MH broth in polystyrene tubes (Ø 17 mm × H 100 mm) (SPL Life Sciences, Korea). The tubes were incubated at different time periods at 30°C without shaking. For biofilm assay, planktonic cells were dried for 30 min. and the number of colonies was counted.
were removed post-incubation and washed thrice with sterile distilled water. For pellicle assay, 100% ethanol was carefully added to the bottom of the pellicle and the pellicle was removed and resuspended in 1 ml of PBS. The pellicle suspension was centrifuged at 13,000 rpm and the supernatant was carefully removed. Both biofilm and pellicle was stained with 0.1% (wt/vol) crystal violet (Sigma, USA) for 15 min at RT followed by washing with sterile distilled water. For pellicle assay, 100% ethanol was carefully removed. For the invasion assay, A549 epithelial cells were seeded into 24-well plates containing DMEM (WELGENE Inc., Korea) supplemented with 10% FBS (WELGENE Inc., Korea) and incubated at 37°C in 5% CO₂ for 24 h. A. nosocomialis WT, acrR deletion mutant and complemented strains were grown in LB broth overnight at 37°C, washed and diluted in DMEM containing FBS. The cell line was infected with different A. nosocomialis strains at a ratio of bacteria to host cells of 100:1 (MOI of 100) and incubated at 37°C in 5% CO₂ for 5 h. After washing five times with Dulbecco’s Phosphate Buffered Saline (DPBS), the cells were fixed with methanol for 20 min, and stained with Giemsa solution (Lee et al., 2006).

### Invasion Assay

For the invasion assay, 1 × 10⁵ A549 epithelial cells were seeded into 24-well plates containing DMEM (WELGENE Inc., Korea) supplemented with 10% FBS (WELGENE Inc., Korea) and incubated at 37°C in 5% (vol/vol) CO₂ for 24 h. Cultures of A. nosocomialis WT, acrR deletion mutant and complemented strains were grown in LB broth overnight at 37°C, washed and diluted in DMEM supplemented with FBS. The cell line was washed with DPBS (WELGENE Inc., Korea) and infected with the bacterial cultures at an MOI of 100. After incubation for 5 h at 37°C in 5% (vol/vol) CO₂, the wells were washed three times with DPBS before adding DMEM-FBS containing 300 µg/ml of

### Table 2 | Oligonucleotides used in this study.

| Oligonucleotides | Sequence (5’ → 3’)<sup>AB</sup> | Purpose |
|------------------|----------------------------------|---------|
| AcrR01F          | AAAACCTCTCTATATACAGTAAAAATTAAT  | Construction of acrR deletion mutant |
| AcrR01R          | AAATATGCGTCTGAGGCGAGAC          | Construction of acrR deletion mutant |
| AcrR02F          | CAAGGCTCTAAATAAGGTCGCC          | Construction of acrR deletion mutant |
| AcrR02R          | GTGGCAAACAGTTAATCAAAGTTAA       | Construction of acrR deletion mutant |
| AcrR03F          | GTGGCAAACAGTTAATCAAAGTTAA       | Construction of acrR deletion mutant |
| AcrR03R          | GTGGCAAACAGTTAATCAAAGTTAA       | Construction of acrR deletion mutant |
| PhaC01F          | AAAGATCAAAGGCACCACAATG          | Construction of acrR deletion mutant |
| PhaC01R          | ATTAATTTAATTTATCACAAAACCATTTT  | Amplification of nptI |
| PhaC02F          | GTGGCAAACAGTTAATCAAAGTTAA       | Confirmation of acrR as polycistronic operon |
| PhaC02R          | GTGGCAAACAGTTAATCAAAGTTAA       | Confirmation of acrR as polycistronic operon |
| PhaC02F          | GTGGCAAACAGTTAATCAAAGTTAA       | For overexpression of AcrR |
| PhaC02R          | GTGGCAAACAGTTAATCAAAGTTAA       | For overexpression of AcrR |
| PhaC01R          | AAAGATCAAAGGCACCACAATG          | For EMSA |
| PhaC02R          | AAAGATCAAAGGCACCACAATG          | For EMSA |
| 16s rDNA_F       | GTTTTTATGACTAAATTAATATTGGTTAACT| Amplification of nptI |
| 16s rDNA_R       | GTTTTTATGACTAAATTAATATTGGTTAACT| Amplification of nptI |
| AcrA_F           | AACACCTCTATATACAGTAAAAATTAAT  | Confirmation of acrR as polycistronic operon |
| AcrA_R           | ATTAATTTAATTTATCACAAAACCATTTT  | For overexpression of AcrR |
| AcrA_F           | AACACCTCTATATACAGTAAAAATTAAT  | For overexpression of AcrR |
| AcrA_R           | ATTAATTTAATTTATCACAAAACCATTTT  | For overexpression of AcrR |
| AcrB_F           | AAAGATCAAAGGCACCACAATG          | For EMSA |
| AcrB_R           | ATTAATTTAATTTATCACAAAACCATTTT  | For EMSA |
| AcrB_F           | AAAGATCAAAGGCACCACAATG          | For EMSA |
| AcrB_R           | ATTAATTTAATTTATCACAAAACCATTTT  | For EMSA |
| csuD_F           | AACACCTCTATATACAGTAAAAATTAAT  | For EMSA |
| csuD_R           | AAAACCTCTCTATATACAGTAAAAATTAAT  | For EMSA |
| csuC_F           | AACACCTCTATATACAGTAAAAATTAAT  | For EMSA |
| csuC_R           | AACACCTCTATATACAGTAAAAATTAAT  | For EMSA |
| csuA_F           | AACACCTCTATATACAGTAAAAATTAAT  | For EMSA |
| csuA_R           | AACACCTCTATATACAGTAAAAATTAAT  | For EMSA |
| nptI_F           | AACACCTCTATATACAGTAAAAATTAAT  | For EMSA |
| nptI_R           | AACACCTCTATATACAGTAAAAATTAAT  | For EMSA |
| nptI_F           | AACACCTCTATATACAGTAAAAATTAAT  | For EMSA |
| nptI_R           | AACACCTCTATATACAGTAAAAATTAAT  | For EMSA |

<sup>A</sup>Regions of oligonucleotides that are not complementary to the corresponding templates are underlined.

<sup>B</sup>Added restriction site sequences are indicated in italics.
gentamicin to kill all external bacteria. The microplates were incubated at 37°C in 5% CO₂ for 2 h. The cells were washed with DPBS, lysed with Ultrapure water (WELGENE Inc., Korea) containing 0.25% Triton X-100 for 20 min at RT and appropriate dilutions were dropped onto LB agar plates. The plates were incubated overnight and CFUs were counted to quantify bacteria surviving intracellularly. To visualize the invasion of bacteria by fluorescence microscopy, cells were seeded on 13 mm glass coverslips and incubated for 24 h. After infection for 5 h, the cells were fixed with 3.7% paraformaldehyde for 10 min and permeabilized with 0.25% Triton X-100. Bacterial cells were labeled with polyclonal anti-rabbit AbOmpA antibody (1:1,000) followed by Alexa Fluor® 594-conjugated goat anti-rabbit IgG antibody (Invitrogen, USA). Actin was stained with Alexa Fluor® 488 phalloidin (Invitrogen, USA) and nucleus with DAPI (6-diamidino-2-phenylindole, Invitrogen, USA). The invasion of A. nosocomialis strains into epithelial cells was observed under Leica DMi8 confocal microscope (Leica Microsystems Ltd. Germany).

Animal Experiments

Seven weeks old female BALB/c mice were used to assess the virulence of the acrR deletion mutant. The mice were obtained from Nara Biotech, Korea and the animals were maintained under specific-pathogen-free conditions according to the Institutional Animal Care and Use Committee (IACUC) of Chungnam National University guidelines (permission number: CNU-00999). The animal experiments were performed in accordance with the guidelines of Korean Food and Drug Administration. For induction of neutropenic mouse model, 7-week-old female BALB/c mice were injected intraperitoneally with 100 mg of cyclophosphamide (CTX, Sigma, USA)/kg of body weight on day 1 and 4 prior to injection of bacterial cells, respectively. The mice were anesthetized and groups containing three were infected intratracheally with 1 × 10⁸ CFU/ml of A. nosocomialis WT, acrR deletion mutant and complemented mutant. One hundred microliters of PBS was used to inject the control mice group. Mice were sacrificed 3 days post-infection, and lungs were harvested to assess the bacterial load. The lungs were homogenized and the cell suspension was serially diluted and spotted onto LB plates to count the bacteria. For histological analysis, tissues were subjected to hematoxylin/eosin, or immunohistochemical staining with OmpA (outer membrane protein A) antibody to visualize the bacteria.

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., USA). *P < 0.05, **P < 0.01, and ***P < 0.001 were considered as statistically significant.

RESULTS

The A. nosocomialis Genome Encodes acrAB Operon Which Is Under the Control of acrR

In this study, in silico analysis revealed the presence of a well-defined acrAB efflux pump operon in A. nosocomialis comprising of acrA, which encodes the membrane protein, and acrB, encoding acriflavine resistance protein (Figure 1A). The acrA and acrB genes of A. nosocomialis showed 24 and 25% identity to those of E. coli, respectively. The gene which encodes the outer membrane protein of the AcrAB efflux pump of A. nosocomialis is yet to be identified. The acrAB efflux pump is under the control of acrR gene upstream of it (Figure 1A) and the acrBR operon and acrR are divergently transcribed under the same promoter activity. We confirmed that acrAB is a polycistronic operon by amplifying the genomic region covering the 3′-end of acrR and 5′-end of acrB using cDNA prepared from DNAse-treated RNA (Figure 1B). The acrR is widely distributed among bacterial species and phylogenetic analysis was carried out to find out the relationships of acrR. The acrR from Acinetobacter strains showed close similarity with that from V. parahaemolyticus RIMD 2210633 and M. haemolytica PHL213 (Figure 1C). Also, the AcrR from A. nosocomialis shared high similarity of 85.38% and 88.41% with that of A. baumannii 17978 and A. baumannii 19606, respectively (Figure 1D). The well-studied AcrR from E. cloacae, E. coli K12 and S. enterica shared similarity of 25.9, 24.31, and 24.86%, respectively.

AcrR Binds to the Promoter Region of acrA/acrR

In E. coli, AcrR functions as one of the regulators modulating the expression of acrAB operon (Ma et al., 1996). In this study, in silico analysis revealed the presence of an AcrR binding motif in the promoter region of acrA/acrR (Figure 2A). In order to examine the direct interaction between AcrR and PacrA/acrR, EMSA was performed with a 148 bp fragment of the promoter region and purified AcrR. With the addition of AcrR, the free DNA substrate disappeared and the DNA bands shifted (Figure 2B), indicating that AcrR could bind to the acrAB regulatory region. The observed interactions were specific because no AcrR binding of a 16S rDNA control fragment was observed (Figure 2B). The AcrR binding motif was located at position −87 (TTTATAAGTTTTTTTAAAAATGATCC) relative to the translational start site (Figure 2A), according to the consensus sequences reported earlier in E. coli (Su et al., 2007). The consensus bases matching with those in the AcrR binding motif in E. coli are underlined. The EMSA data clearly shows that AcrR specifically binds to the promoter region of acrA/acrR and thus suggests an involvement in the regulation of expression of acrAB operon.

AcrR Negatively Regulates acrAB Expression

Previous studies have pointed out that the expression of acrAB operon is downregulated by the local repressor AcrR in E. coli.
FIGURE 1 | Genomic organization of acrAB operon and in silico analysis of acrR. (A) The schematic representation of the acrAB efflux pump operon in A. nosocomialis. The acrR gene which is divergently transcribed from the acrAB operon shares a common promoter region with the acrAB operon. (B) Confirmation of acrAB as polycistronic operon. RT-PCR analysis was performed using total RNA isolated from A. nosocomialis wild type strain grown in LB medium at 37°C. A negative control and positive control reactions were performed using DNase-treated RNA and genomic DNA as templates, respectively. The size of the amplified fragments was estimated by comparing it with the size marker DNAs (lane M). (C) Phylogenetic analysis of acrR from A. nosocomialis with that of other bacterial strains. The tree was constructed using MEGA 6.06 and the pairwise distance was calculated using Maximum Likelihood approach according to the aligned sequences. (D) Multiple sequence alignment of AcrR from A. nosocomialis with homologs from different bacterial strains. Residues that are conserved across all sequences are highlighted in dark gray and matches across the sequences by light gray. Pairwise sequence identity (%) is also shown. A.bau 17978, A. baumannii 17978; A.nos 13TU, A. nosocomialis; A.bau 19606; A. baumannii 19606.

and S. Typhimurium (Olliver et al., 2004; Pourahmad Jaktaji and Jazayeri, 2013). In A. nosocomialis, the DNA-binding assay revealed an interaction of AcrR with the promoter region of acrA/acrR. To functionally analyze the effect of AcrR on acrAB expression in A. nosocomialis, RT-PCR transcription analyses were performed using wild type and acrR mutant strains. Deletion of acrR increased acrA and acrB expression in the mutant compared to the wild type (Figure 3). The data demonstrates that AcrR negatively regulates the expression of acrAB operon, similar to that in E. coli and S. Typhimurium.

Deleting acrR Enhances Motility

The acrR deletion mutant of A. nosocomialis ATCC 17903 was constructed using markerless gene deletion method based on double cross over. The detailed protocol for the construction of deletion mutant is given in the “Materials and Methods” section. The whole acrR gene was deleted and the deletion was confirmed by PCR (Figure 4A) followed by sequencing. For the complementation of acrR mutant, the acrR coding region along with its native promoter was inserted into intergenic region that is located downstream of phuC encoding alpha/beta hydrolase. The complementation of the acrR mutant was confirmed by PCR. To characterize the acrR mutant, A. nosocomialis wild type, acrR mutant and complemented mutant were cultivated in MH broth and the OD600 was measured until 24 h of incubation. It was noticed that the growth pattern of acrR mutant was not different from that of wild type.

It has been reported previously that AcrR modulates motility in E. coli (Kim et al., 2016). To check the effect of acrR deletion on motility in A. nosocomialis, overnight cultures of A. nosocomialis WT, acrR deletion mutant and complemented mutant were spotted on MH medium containing 0.25% Eiken soft agar. The
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FIGURE 2 | EMSA analysis for the AcrR interaction with the acrR-acrAB intergenic region. (A) Scheme and nucleotide sequence of the intergenic region of acrR and acrAB. A putative AcrR binding motif located in the promoter region between acrR and acrA is shown. The number beneath the indicated binding site of AcrR points the position of the first nucleotide in the binding motif relative to the translational start site of acrA. (B) EMSA result showing the interaction between AcrR and the promoter region of acrA/acrR. The promoter region and 16s rDNA (as control) was amplified by PCR and incubated without or with different concentrations of purified AcrR. 1 µg of poly[d(I-C)] was added to each reaction mixture in order to avoid non-specific binding.

data showed that motility of the acrR mutant is significantly increased compared to that of wild type, indicating that AcrR plays an important role in the regulation of motility in A. nosocomialis (Figure 4B). As expected, the acrR complemented strain restored the motility similar to that of the wild type strain.

Deletion of acrR Increases Biofilm/Pellicle Formation

Multidrug efflux pumps are known to be important constituent of biofilm formation (Lynch et al., 2007; Kvist et al., 2008; Matsumura et al., 2011; Baugh et al., 2012). In S. enterica serovar Typhimurium, the mutation in the acrAB operon led to impaired biofilm formation (Baugh et al., 2014; Schlisselberg et al., 2015). Similarly, the acrB mutant of E. coli also depicted decreased biofilm formation (Kvist et al., 2008; Matsumura et al., 2011). Therefore, we checked the effect of acrR deletion on biofilm/pellicle formation in A. nosocomialis by cultivating the strains in MH broth at 30°C over a period of time. Crystal violet staining was carried out to quantify separately the biofilm attached to the culture tubes and the pellicle formed at the air-liquid interface. acrR mutant exhibited increased biofilm formation even though the growth rate of the mutant was
AcrR Deletion Enhances Cell Adhesion and Invasion

Multidrug efflux pumps including AcrAB-TolC efflux system play an important role in the adherence and invasiveness of pathogenic bacteria to the host cells (Hirakata et al., 2002; Buckley et al., 2006). We determined the adherence and invasiveness of acrR mutant to A549 epithelial cells and noticed higher adherence and invasion ability compared to the wild type (Figure 6, Supplementary Figure 1). The adherence and invasiveness of the complemented mutant was similar to that of the wild type. The data depicts that AcrR plays a negative role in the adherence and invasiveness of A. nosocomialis. We checked the expression level of the genes, csuC and csuD which encodes components of the Csua/BABCDE chaperone-usher pilus assembly system important for the assembly and production of pilus involved in adhesion in Acinetobacter by mRNA expression analysis. It was demonstrated that the expression of csuC and csuD genes are upregulated in the acrR deletion mutant compared to the wild type (Supplementary Figure 2). Thus, it can be concluded that the elevated expression of csuC and csuD might contribute to the increased adhesion and invasion in the acrR mutant.

acrR Deletion Mutant Displays Increased Virulence

It has been reported previously that the AcrAB-TolC efflux system plays a direct role in the virulence of pathogenic bacteria. In S. Typhimurium, acrB insertionally inactivated/deleted mutant displayed less virulence compared to the wild type strain (Buckley et al., 2006; Perrett et al., 2009; Webber et al., 2009). To investigate the role of AcrR on virulence, we infected BALB/c mice with wild type, acrR mutant and complemented mutant of A. nosocomialis intratracheally. Survival curve was plotted to assess the overall virulence of the mutant compared to the wild type. As shown in Figure 7A, the mice infected with the acrR mutant had a significantly lower survival rate. The CFUs of the mutant in the lungs of infected mice were 1.5 times higher than that of wild type in the infected lungs (Figure 7B). In addition, H&E staining and IHC of the lung tissues revealed that the acrR mutant severely damaged the lungs compared to the wild type (Figure 7C). The lung tissues from acrR mutant depicted increased cellular infiltration compared to that of the wild type. The IHC data showed higher number of bacterial cells in the damaged lung tissues from the acrR mutant than from the wild type tissues. The
above results demonstrate that AcrR suppresses virulence in *A. nosocomialis* and thus the *acrR* mutant is highly pathogenic than the wild type.

**DISCUSSION**

Multidrug efflux pumps have been recognized as playing a pivotal role in drug resistance, cell division, pathogenicity and biofilm formation (Nikaido, 1996; Buckley et al., 2006; Kvist et al., 2008). Here, we identified an operon encoding AcrA and AcrB in *A. nosocomialis*, which shows significant homology to the AcrAB efflux pump of *E. coli*. The *acrAB* operon in *A. nosocomialis* depicted high similarity to the *arpAB* operon which plays a role in aminoglycoside resistance in *A. baumannii* 5075 (Tipton et al., 2017). In *E. coli*, the *acrAB* operon is regulated by AcrR, encoded by *acrR* located 140 bp upstream of the *acrAB* operon (Ma et al., 1996). The AcrR belongs to TetR family of transcriptional regulators and is composed of an N-terminal domain containing a DNA-binding helix-turn-helix (HTH) motif, and a C-terminal domain containing an unique ligand-binding sequence (Ramos et al., 2005; Li et al., 2007; Su et al., 2007). *In silico* analysis revealed that the DNA-binding motif of AcrR is highly conserved in the bacterial community (data not shown). Similar to that in *E. coli*, the *acrR* in *A. nosocomialis* is located upstream of *acrAB* operon and is divergently transcribed from the same promoter of *acrA*. Also, the AcrR binding motif identified in the PacrA/acrR of *A. nosocomialis* showed close similarity to that in *E. coli*.

*In silico* analysis and further confirmation by EMSA revealed that AcrR of *A. nosocomialis* binds to the promoter region...
of acrA/acrR and might control the expression of acrAB. The expression of acrAB was elevated in the acrR mutant, which was confirmed by RT-PCR. In E. coli, AcrR negatively modulates the expression of the AcrAB efflux pump and the absence of functional AcrR resulted in increased transcription of acrAB (Ma et al., 1996). In addition, AcrR acts as an autorepressor regulating its own gene expression in E. coli.

It was found that deletion of acrR leads to increased motility in A. nosocomialis, which is in accordance with the motility observed in the acrR mutant of E. coli (Kim et al., 2016). In E. coli, the deletion of acrR induces transcription of most motility genes, indicating that the toxic-compounds-response regulator AcrR participates in motility to escape toxic compounds. The inactivation of acrB in S. enterica negatively impacts survival in the host and motility (Webber et al., 2009) contributing to reduced pathogenicity (Khoramian-Falsafi et al., 1990). The data from our study reveal that AcrR has a negative role to play in motility by controlling the expression of AcrAB efflux pump.

The absence of the AcrAB efflux pump repressor, AcrR contributed to increased biofilm/pellicle formation, indicating that the AcrAB efflux pump is positively associated with biofilm formation in A. nosocomialis. It has been reported previously that a number of multidrug efflux systems, including AcrAB-TolC, play a role in biofilm formation in various Gram negative species, including E. coli, Klebsiella and S. Typhimurium (Kvist et al., 2008; Baugh et al., 2012). In addition, an upregulated expression of multidrug efflux genes, acrA and acrB has been noticed in biofilm cells of E. coli (Zou et al., 2012). The study that explores the functional link between multidrug efflux pump and biofilm formation in S. Typhimurium revealed that several multidrug efflux pumps, including AcrAB plays a role in the formation of extracellular matrix (Baugh et al., 2014). It has been known that the extracellular biofilm matrix of E. coli and Salmonella is majorly composed of structural curli proteins which help them in adhesion to inert surfaces as well as to host cells (Cegelski et al., 2009; Baugh et al., 2014). The curli proteins are constituted by the major and minor curli subunits which are synthesized by the csgBA operon (Grund and Weber, 1988; Andersson et al., 2013). It has been noticed previously that the acrB and tolC mutants of S. Typhimurium lack the production of curli due to the transcriptional repression of csgA and csgB genes (Baugh et al., 2014), indicating the importance of AcrAB efflux pump in curli production.

It has been known for decades that there is a significant correlation between biofilm formation and multidrug resistance (Anderl et al., 2000). The bacterial cells which are associated to biofilm can tolerate antibacterial agents far better than planktonic cells. The MexAB-OprM and MexCD-OprJ pumps of P. aeruginosa have been shown to be involved in biofilm-specific mechanisms for resistance (Gillis et al., 2005). In addition, the efflux pump operon, PA1874-1877 in P. aeruginosa was seen upregulated during biofilm formation and that appeared to be involved in biofilm-mediated antibiotic resistance (Zhang and Mah, 2008). Further studies on the expression level of AcrAB efflux pump and multidrug resistance under biofilm-forming conditions would shed light on the involvement of this pump in biofilm-mediated antibiotic resistance in A. nosocomialis.

The invasiveness and pathogenesis of A. nosocomialis was elevated when the acrR gene was deleted. Earlier studies have shown that the AcrAB-TolC efflux system and its homologs are crucial for the invasion/virulence of S. Typhimurium, Moraxella catarrhalis, P. aeruginosa, and Erwinia amylovora (Hirakata et al.,...
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FIGURE 7 | Deletion of acrR promotes virulence in A. nosocomialis. To investigate the effect of acrR deletion on the virulence of A. nosocomialis, mice were intratracheally infected with A. nosocomialis wild type (WT), acrR mutant (ΔacrR) and complemented mutant (C-ΔacrR). (A) Survival curve of mice infected with different bacterial strains. The lethality was higher in the case of mice infected with acrR mutant compared to the wild type. (B) Bacterial burden in lungs infected with A. nosocomialis strains. Lungs were homogenized 3 days post-infection and CFU counting was performed. (C) H&E, IHC staining results showing severe tissue damage in lungs of mice infected with acrR mutant compared to that with wild type. Values are mean ± SD (n = 3) and asterisks indicate a significant difference in CFU value compared with the wild type at **P < 0.01. Scale bar, 500 µm.

The deletion of acrR led to the upregulation of csuC and csuD genes which are important for the production and assembly of the virulence factor pili needed for adhesion in A. nosocomialis. Based on the fact that acrAB operon is induced in the acrR mutant, it could be suggested that the overexpression of the AcrAB efflux pump might play a role in the enhanced production of virulence factors contributing to increased biofilm formation, adhesion, invasion and pathogenesis in A. nosocomialis.

The involvement of multidrug efflux pumps in quorum sensing (QS) was first reported in P. aeruginosa in which the MexAB-OprM plays a role in the efflux of autoinducer N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) (Pearson et al., 1999). The mexAB-oprM mutant displayed reduced diffusion of the 3-oxo-C12-HSL into the extracellular environment resulting in loss of virulence. The transcription of quorum sensing transporter genes lsrACDBK and the operon repressor lsrR was decreased in the acrB mutant of S. Typhimurium (Wang-Kan et al., 2017), indicating the role of AcrAB in the expression of virulence factors as lsrR controls the expression of SPI-related genes (Choi et al., 2012). In Burkholderia pseudomallei, the extracellular secretion of AHLs...
is absolutely dependent on the function of BpeAB-OprB efflux pump (Chan et al., 2007). Interestingly, in our study, RNA sequencing and mRNA expression analysis of the anoR mutant of *A. nosocomialis* depicted the downregulation of *acrA* and *acrB* genes (data unpublished). It has been reported earlier that the expression of **mexAB-oprM** of *P. aeruginosa* is limited by the intracellular concentration of the autoinducer molecules (Maseda et al., 2004). Thus, the decreased expression of *acrA* and *acrB* in the anoR mutant could be very well-associated with the absence of AHLs in the mutant background. It is very well-known that QS plays a crucial role in motility, biofilm formation and the production of virulence factors (Rutherford and Bassler, 2012). Thus, the enhanced AcrAB efflux pump activity in the anoR mutant might attribute well to the AHLs-mediated cell-to-cell signaling, contributing to increased motility, biofilm formation and virulence in the mutant.

In summary, we report a novel regulator, AcrR which negatively regulates the uncharacterized multidrug efflux operon, **acrAB** in *A. nosocomialis*. The bacterial motility, biofilm/pellicle formation and virulence were increased in the *acrR* mutant with the upregulated expression of **acrAB** operon. This supports the notion that besides contributing to antibiotic resistance, multidrug efflux pumps display a variety of functions in accordance to the environment and the bacterial behavior. Further studies are ongoing for in-depth characterization of the AcrAB efflux pump so that the knowledge could be used for developing efflux pump inhibitors as they are invaluable tools to restore the activity of agents to which the efflux pump confers resistance and also to reduce the ability of bacteria to infect their host.

**AUTHOR CONTRIBUTIONS**

BS, JK, MO, and CC designed the research. BS, JK, DK, KW, and MO performed the research. BS, JK, DK, KW, MO, and CC analyzed the data. BS, MO, and CC wrote the paper.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2018.00270/full#supplementary-material

**REFERENCES**

Alves, C., and Cunha, C. (2012). “Electrophoretic mobility shift assay: analyzing protein - nucleic acid interactions,” in *Gel Electrophoresis*, ed S. Magdeldin (IntechOpen), 205–228. doi: 10.5772/37619

Anderl, J. N., Franklin, M. J., and Stewart, P. S. (2000). Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. *Antimicrob. Agents Chemother.* 44, 1818–1824. doi: 10.1128/AAC.44.7.1818-1824.2000

Andersson, E. K., Bengtsson, C., Evans, M. L., Chorrell, E., Sellstedt, M., Lindgren, A. E., et al. (2013). Modulation of curli assembly and pellicle biofilm formation by chemical and protein chaperones. *Chem. Biol.* 20, 1245–1254. doi: 10.1016/j.chembiol.2013.07.017

Antunes, L. C., Visca, P., and Towner, K. J. (2014). *Acinetobacter baumannii*: evolution of a global pathogen. *Pathog. Dis.* 71, 292–301. doi: 10.1111/2049-632X.12125

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 *Typhi*onium results in impaired ability to form a biofilm. *J. Antimicrob. Chemother.* 67, 2409–2417. doi: 10.1093/jac/dks228

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. doi: 10.1093/jac/dkt420

Bina, X. R., Provenzano, D., Nguyen, N., and Bina, J. E. (2008). Vibrio cholerae RND family efflux systems are required for antimicrobial resistance, optimal virulence factor production, and colonization of the infant mouse small intestine. *Infect. Immun.* 76, 3595–3605. doi: 10.1128/IAI.01620-07

Buckley, A. M., Webber, M. A., Cools, S., Randall, L. F., La Ragione, R. M., Woodward, M. J., et al. (2006). The AcrAB-ToLC efflux system of *Salmonella enterica* serovar *Typhi*onium plays a role in pathogenesis. *Cell. Microbiol.* 8, 847–856. doi: 10.1111/j.1462-5822.2005.00671.x

Bunikis, I., Denker, K., Ostberg, Y., Andersen, C., Benz, R., and Bergström, S. (2008). An RND-type efflux system in *Borrelia burgdorferi* is involved in virulence and resistance to antimicrobial compounds. *PLoS Pathog.* 4:e1000009. doi: 10.1371/journal.ppat.1000009

Burse, A., Weingart, H., and Ullrich, M. S. (2004). The phytoalexin-inducible multidrug efflux pump AcrAB contributes to virulence in the fire blight pathogen, *Erwinia amylovora*. *Mol. Plant. Microbe Interact.* 17, 43–54. doi: 10.1094/MPMI.2004.17.1.43

CDC (2013). *Antibiotic Resistance Threats in the United States 2013*.

Cegelski, L., Pinkner, J. S., Hammer, N. D., Casumano, C. K., Hung, C. S., Chorrell, E., et al. (2009). Small-molecule inhibitors target *Escherichia coli* amyloid biogenesis and biofilm formation. *Nat. Chem. Biol.* 5, 913–919. doi: 10.1038/nchembio.242

Chan, Y. Y., Bhan, H. S., Tan, T. M., Mattmann, M. E., Geske, G. D., Igarashi, J., et al. (2007). Control of quorum sensing by a *Burkholderia pseudomallei* multidrug efflux pump. *J. Bacteriol.* 189, 4320–4324. doi: 10.1128/JB.0003-07

Choi, J., Shin, D., Kim, M., Park, J., Lim, S., and Ryu, S. (2012). LsrR-mediated quorum sensing controls invasiveness of *Salmonella typhimurium* by regulating SPI-1 and flagella genes. *PLoS ONE* 7:e37059. doi: 10.1371/journal.pone.0037059

Cosgaya, C., Mari-Almirall, M., Van Asche, A., Fernández-Orth, D., Mosqueda, N., Telli, M., et al. (2016). *Acinetobacter dykshoorniae* sp. nov., a member of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex mainly recovered from clinical samples in different countries. *Int. J. Syst. Evol. Microbiol.* 66, 4105–4111. doi: 10.1099/ijsem.0.001318

Coyne, S., Gui gon, G., Courvalin, P., and Périchon, B. (2010a). Screening and quantification of the expression of antibiotic resistance genes in *Acinetobacter baumannii* with a microarray. *Antimicrob. Agents Chemother.* 54, 333–340. doi: 10.1128/AAC.00137-09

Coyne, S., Rosenfeld, N., Lambert, T., Courvalin, P., and Périchon, B. (2010b). Overexpression of resistance-nodulation-cell division pump AdeFGH confers multidrug resistance in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* 54, 4389–4393. doi: 10.1128/AAC.00155-10
Acinetobacter baumannii: evolution of antimicrobial resistance-treatment options. Semin. Respir. Crit. Care Med. 36, 85–98. doi: 10.1055/s-0034-1398388

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32, 1792–1797. doi: 10.1093/nar/gkh340

Fournier, P. E., and Richet, H. (2006). The epidemiology and control of Acinetobacter baumannii in health care facilities. Clin. Infect. Dis. 42, 692–699. doi: 10.1086/500202

Giammanco, A., Calà, C., Fasciana, T., and Dowzicky, M. J. (2017). Global assessment of the activity of tigecycline against multidrug-resistant Gram-Negative pathogens between 2004 and 2014 as part of the tigecycline evaluation and surveillance trial. mSphere 2:e00310-16. doi: 10.1128/mSphere.00310-16

Grund, S., and Weber, A. (1988). A new type of fimbriae on Salmonella typhimurium. J. Vet. Med. 35, 779–782. doi: 10.1111/j.1439-0450.1988.tb0560.x

Hanahan, D. (1983). Studies on transformation of Escherichia coli with plasmids. J. Mol. Biol. 166, 557–580. doi: 10.1016/0022-2836(83)90284-8

Hirakata, Y., Srikumar, R., Poole, K., Gotoh, N., Suematsu, T., Kohn, S., et al. (2002). Multidrug efflux systems play an important role in the invasiveness of Pseudomonas aeruginosa. J. Exp. Med. 196, 109–118. doi: 10.1084/jem.20020005

Khoramian-Falsafi, T., Harayama, S., Kutsukake, K., and Pechère, J. C. (1990). AcrR, the local repressor of AcrAB, plays a modulating role in the regulation of acrAB genes of Escherichia coli by global stress signals. Mol. Microbiol. 19, 101–112. doi: 10.1111/j.1469-0939.1996.037881.x

Köhler, T., Michéa-Hamzehpour, M., Henze, U., Gotoh, N., Poole, K., Yamashita, E., Matsumoto, T., and Yamaguchi, A. (2009). Regulation and physiological function of multidrug efflux pumps in the biofilm formation of Escherichia coli strain BM4545. Antimicrob. Agents Chemother. 45, 3375–3380. doi: 10.1128/AAC.01375-08

Marchand, I., Damier-Piolle, L., Courvalin, P., and Lambert, T. (2004). Expression of the RND-type efflux pump AdeABC in Acinetobacter baumannii is regulated by the AdeRS two-component system. Antimicrob. Agents Chemother. 48, 3298–3304. doi: 10.1128/AAC.48.9.3298-3304.2004

Matsutmura, K., Furukawa, S., Oghara, H., and Morinaga, Y. (2011). Multidrug efflux pumps on the biofilm formation of Escherichia coli K-12. Biocontrol Sci. 16, 69–72. doi: 10.4265/bio.16.69

Murakami, S., Nakashima, R., Yamashita, E., Matsumoto, T., and Yamaguchi, A. (2006). Crystal structures of a multidrug transporter reveal a functionally rotating mechanism. Nature 443, 173–179. doi: 10.1038/nature05076

Murakami, S., Nakashima, R., Yamashita, E., and Yamaguchi, A. (2002). Crystal structure of bacterial multidrug efflux transporter AcrB. Nature 419, 587–593. doi: 10.1038/nature01050

Nemec, A., Krizova, L., Maixnerova, M., Sedo, O., Briese, S., and Higgins, P. G. (2015). Acinetobacter seifertii sp. nov., a member of the Acinetobacter calcoaceticus-Acinetobacter baumannii complex isolated from human clinical specimens. Int. J. Syst. Evol. Microbiol. 65, 934–942. doi: 10.1099/ijsem.0.009043

Nemec, A., Krizova, L., Maixnerova, M., van der Reijden, T. J., Deschaght, P., Passet, V., et al. (2011). Genotypic and phenotypic characterization of the Acinetobacter calcoaceticus-Acinetobacter baumannii complex with the proposal of Acinetobacter pittii sp. nov. (formerly Acinetobacter genomic species 3) and Acinetobacter nosocomialis sp. nov. (formerly Acinetobacter genomic species 13TU). Res. Microbiol. 162, 393–404. doi: 10.1016/j.resmic.2011.02.006

Nikaido, H. (1996). Multidrug efflux pumps of gram-negative bacteria. J. Bacteriol. 178, 5853–5859. doi: 10.1128/jb.178.20.5853-5859.1996

Nishino, K., Nikaido, E., and Yamaguchi, A. (2009). Regulation and physiological function of multidrug efflux pumps in Escherichia coli and Salmonella. Biochim. Biophys. Acta 1794, 834–843. doi: 10.1016/j.bbapap.2009.02.002

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 anoIR gene. J. Microbiol. Biotechnol. 25, 1390–1400. doi: 10.4136/jmb.1504.04069

Okusu, H., Ma, D., and Nikaido, H. (1996). AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of Escherichia coli multiple-antibiotic-resistant (Mar) mutants. J. Bacteriol. 178, 306–308. doi: 10.1128/jb.178.1.306-308.1996
Olliver, A., Valle, M., Chaslus-Dancla, E., and Cloeckaert, A. (2004). Role of an acrR mutation in multidrug resistance of in vitro-selected fluoroquinolone-resistant mutants of Salmonella enterica serovar Typhimurium. FEMS Microbiol. Lett. 238, 267–272. doi: 10.1111/j.1574-6968.2004.tb09766.x

Paulsen, I. T., Chen, J., Nelson, K. E., and Saier, M. H. J. (2001). Comparative genomics of microbial drug efflux systems. J. Mol. Microbiol. Biotechnol. 3, 145–150.

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.

Peleg, A. Y., and Hooper, D. C. (2010). Hospital-acquired infections due to gram-negative bacteria. N. Engl. J. Med. 362, 1804–1813. doi: 10.1056/NEJMra0904124

Peleg, A. Y., Seifert, H., and Paterson, D. L. (2008). Acinetobacter baumannii: emergence of a successful pathogen. Clin. Microbiol. Rev. 21, 538–582. doi: 10.1128/CMR.00058-07

Perrett, C. A., Karavolos, M. H., Humphrey, S., Mastroeni, P., Martinez-Ardou, I., Spencer, H., et al. (2009). LuxS-based quorum sensing does not affect the ability of Salmonella enterica serovar Typhimurium to express the SPI-1 type 3 secretion system, induce membrane ruffles, or invade epithelial cells. J. Bacteriol. 191, 7253–7259. doi: 10.1128/JB.00727-09

Piddock, L. J. (2006). Multidrug-resistance efflux pumps - not just for resistance. Nat. Rev. Microbiol. 4, 629–636. doi: 10.1038/nrmicro1464

Poole, K. (2007). Efflux pumps as antimicrobial resistance mechanisms. Ann. Med. 39, 162–176. doi: 10.1080/07853890701195262

Poole, K., Gotoh, N., Tsujimoto, H., Zhao, Q., Wada, A., Yamashita, T., et al. (1996). Overexpression of the mexC-mexD-opr efflux operon in nfsB-type multidrug-resistant strains of Pseudomonas aeruginosa. Mol. Microbiol. 21, 713–724. doi: 10.1046/j.1365-2958.1996.261397.x

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. doi: 10.1128/JB.175.22.7363-7372.1993

Pourahmad Jaktaji, R., and Jazayeri, N. (2013). Expression of acrA and acrB Genes in Acinetobacter baumannii and its Clinical Significance. Iran. J. Med. Sci. 8, 619–625.

Piddock, L. J. (2006). Multidrug-resistance efflux pumps - not just for resistance. Nat. Rev. Microbiol. 4, 629–636. doi: 10.1038/nrmicro1464

Poole, K., Gotoh, N., Tsujimoto, H., Zhao, Q., Wada, A., Yamashita, T., et al. (1996). Overexpression of the mexC-mexD-opr efflux operon in nfsB-type multidrug-resistant strains of Pseudomonas aeruginosa. Mol. Microbiol. 21, 713–724. doi: 10.1046/j.1365-2958.1996.261397.x

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. doi: 10.1128/JB.175.22.7363-7372.1993

Pourahmad Jaktaji, R., and Jazayeri, N. (2013). Expression of acrA and acrB Genes in Acinetobacter baumannii and its Clinical Significance. Iran. J. Med. Sci. 8, 619–625.

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 C. jejuni: evidence for involvement of an efflux operon. J. Bacteriol. 186, 326–356. doi: 10.1128/JB.186.2.326-356.2004

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