Contribution of *Acinetobacter*-derived cephalosporinase-30 to sulbactam resistance in *Acinetobacter baumannii*

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The sulbactam resistance rate in *Acinetobacter baumannii* has increased worldwide. Previous reports have shown that the β-lactamase bla_{TEM-1} confers resistance to sulbactam in *A. baumannii*. The purpose of this study was to examine whether other β-lactamases, including the *Acinetobacter*-derived cephalosporinase (ADC), OXA-23, OXA-24/72, and OXA-58 families, also contribute to sulbactam resistance in *A. baumannii*. The correlation between these β-lactamases and the sulbactam minimal inhibitory concentration (MIC) was determined using *A. baumannii* clinical isolates from diverse clonality, which were collected in a nationwide surveillance program from 2002 to 2010 in Taiwan. A possible association between the genetic structure of IS*Aba1*-bla_{ADC-30} and sulbactam resistance was observed because this genetic structure was detected in 97% of sulbactam-resistant strains compared with 10% of sulbactam-susceptible strains. Transformation of IS*Aba1*-bla_{ADC-30} into susceptible strains increased the sulbactam MIC from 2 to 32 μg/ml, which required bla_{ADC-30} overexpression using an upstream promoter in IS*Aba1*. Flow cytometry showed that ADC-30 production increased in response to sulbactam, ticarcillin, and ceftazidime treatment. This effect was regulated at the RNA level but not by an increase in the bla_{ADC-30} gene copy number as indicated by quantitative PCR. Purified ADC-30 decreased the inhibitory zone created by sulbactam or ceftazidime, similarly to TEM-1. In conclusion, ADC-30 overexpression conferred resistance to sulbactam in diverse clinical *A. baumannii* isolates.

Keywords: sulbactam, mechanisms of resistance, *Acinetobacter baumannii*, transformation, *Acinetobacter*-derived cephalosporinase (ADC)

Introduction

Acinetobacter baumannii causes various nosocomial infections, and the prevalence of multidrug-resistant (MDR) *A. baumannii* has been increasing in different countries. This bacterium has intrinsic resistance to multiple drugs and can gain resistance mechanisms from other species (Peleg et al., 2008). The SENTRY program documented non-susceptibility to carbapenems, the last resort of drugs for the treatment of MDR *A. baumannii*, increased from 34.5% in 2006 to 59.8% in 2009 worldwide (Gales et al., 2011). In Taiwan, the rate of multidrug resistance in *Acinetobacter spp.* also increased from 1.3% in 2002 to 41.0% in 2010 (Kuo et al., 2012). In severely ill patients, infections with MDR isolates have been associated with high mortality due to the absence of appropriate or effective treatment options (Peleg et al., 2008). Combination therapies or new drugs such as antimicrobial peptides or silver nanoparticles have been proposed as novel modalities to treat MDR *A. baumannii* (Peleg et al., 2008; Tiwari et al., 2014)

Sulbactam is a β-lactamase inhibitor that is typically combined with penicillins because sulbactam lacks antimicrobial activity against most bacterial species (Adnan et al., 2013). However, sulbactam has demonstrated bacteriostatic or bactericidal effects against *A. baumannii* (Corbella et al., 1998). Combination treatment with sulbactam and carbapenems has shown promising *in vivo* and *in vitro* synergistic effects against MDR *A. baumannii* (Wolff et al., 1999; Ko et al., 2004; Song et al., 2007), and clinical success has been reported (Karageorgopoulos and Falagas, 2008). The addition of sulbactam to other antibiotics has been proposed in the treatment of MDR *A. baumannii*; however, the resistance rate to ampicillin/sulbactam in *Acinetobacter spp.* has increased to approximately 60% in certain area (Kuo et al., 2012).

Until recently, the mechanism underlying sulbactam resistance in *A. baumannii* was less commonly studied. In 2013, Krizova and colleagues demonstrated that the β-lactamase TEM-1 contributes to sulbactam resistance (Krizova et al., 2013), which led us to examine whether other selected β-lactamases found in *A. baumannii*, including the *Acinetobacter*-derived cephalosporinase (ADC), OXA-23, OXA-24/72, and OXA-58 families, also contribute to sulbactam resistance. Using clinical isolates collected from a Taiwanese surveillance program, we aimed to identify the β-lactamases associated with sulbactam resistance and to test the role of these β-lactamases in sulbactam resistance in *A. baumannii*.

Materials and Methods

Association of Selected β-Lactamases with Sulbactam Resistance in *A. baumannii*

A. baumannii clinical isolates were randomly selected from the Taiwan Surveillance of Antimicrobial Resistance (TSAR) program, which contains 1640 *Acinetobacter* isolates collected from 2002 to 2010 (Kuo et al., 2012). *A. baumannii* was identified at the species level using multiplex PCR targeting the specific 16-23S rDNA intergenic spacer region (Chen et al., 2007). Pulsed-field gel electrophoresis was performed to determine

clonality as previously described (Kuo et al., 2013). Isolates with similarity of >80% was designated as a single clone. The sulbactam-resistant and sulbactam-susceptible isolates were randomly selected and subjected to PCR testing for the presence of genes encoding ADC, OXA-23, OXA-24/72, and OXA-58 β-lactamases (Table S1). The PCR program (Krizova et al., 2013; Kuo et al., 2013) for genes encoding OXA was as followed: 94°C for 1 min, and 30 cycles of 25 s at 94°C, 40 s at 52°C and 50 s at 72°C; for PCR of bla_{ADC}, 30 cycles of 60 s at 94°C, 60 s at 58°C, and 120 s at 72°C. GoTaq Flexi DNA polymerase (Promega, Madison, WI) was used for PCR assays performed in the GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA). Amplified DNA product was resolved by electrophoresis in agarose 2% w/v gels, stained with ethidium bromide, and purified according to the manufacturer's instruction (Geneaid Biotech Ltd, Taipei, Taiwan).

Transformation of Plasmids Carrying Different β-Lactamase Genes

Representative β-lactamase genes and their associated promoters, including bla_{TEM-1}, bla_{ADC} and bla_{OXA-23} with their upstream insertion sequence IS*Aba1* (IS*Aba1-bla*$_{ADC}$ and IS*Aba1-bla*$_{OXA-23}$, respectively), $bla_{OXA-24/72}$, and bla_{OXA-58} with its upstream IS*Aba3* that was truncated with IS*1008* (IS*1008*-ΔIS*Aba3-bla*$_{OXA-58}$) (Chen et al., 2010), were PCR-amplified using the forward and reverse primers shown in Table S1. The PCR products were amplified with a proof-reading DNA polymerase (Phusion High-Fidelity DNA Polymerase, Finnzymes, Espoo, Finland), cloned into the pCRII-TOPO vector (Invitrogen, Carlsbad, CA, USA) and subjected to sequencing (Mission Biotech, Taipei, Taiwan). The digested fragments were cloned into the *Xba*I and *Xho*I sites of the *Escherichia coli-A. baumannii* shuttle vector pYMAb2 (Kuo et al., 2013), which contains a kanamycin-resistant determinant. The fragment was cloned in-frame with a polyhistidine (His) tag, causing the resulting protein to be His-tagged. The recombinant plasmid and a control plasmid (pYMAb2 without β-lactamase genes) were transformed into the kanamycin-susceptible *A. baumannii* strain ATCC15151. ATCC15151 already contained bla_{OXA-51}; therefore, bla_{OXA-51} was not included in the experiment. Electroporation was performed with a gene pulser electroporator (Bio-Rad, Hercules, CA, USA) and 2-mm electrode gap cuvettes (Kuo et al., 2013). Transformants were selected based on kanamycin resistance, and sequencing was performed to confirm the presence of each β-lactamase gene.

Antimicrobial Susceptibility

Minimal inhibitory concentrations (MICs) of sulbactam, ceftazidime, ampicillin, imipenem, meropenem, and ticarcillin were determined by the agar dilution method according to the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI) (Clinical and Laboratory Standards Institute, 2012). Sulbactam susceptibility parameters were adopted from the previous CLSI guidelines in which sulbactam MICs of less than 4 or more than 16 μg/ml were defined as susceptible or resistant, respectively.

Immunofluorescent Staining and Enumeration by Flow Cytometry for Protein Expression

Immunofluorescent staining was performed as previously described with several modifications (Moe et al., 1999). Specifically, bacterial cultures were diluted in phosphate buffered saline (PBS) to ~10^8 CFU/mL, and 0.5-ml samples were transferred into 1.5-ml Eppendorf microtubes. The samples were centrifuged at 5000 × g for 5 min, washed with 0.1% NaN$_3$ PBS, and centrifuged for 5 min. The resulting bacterial pellet was resuspended in fixative (4% paraformaldehyde in PBS) for 20 min. The fixed samples were washed twice with quenching solution (100 mM NaCl, 50 mM Tris-HCl, pH 8.0) and resuspended in permeable buffer (1% Triton X-100, 0.1% NaN$_3$ in PBS) for 5 min. After centrifugation, the samples were resuspended in 500 μL blocking buffer (1% BSA and 0.1% NaN$_3$ in PBS).

To identify His-tagged ADC-30 expressed by ATCC15151 (pYMAb2::ISAba1-bla$_{ADC-30}$), each 100-μL sample was incubated with 2 μL of mouse anti-His-6-tag antibody (Sigma-Aldrich, St. Louis, MO, USA) at 4°C for 2 h. After washing by blocking buffer, the samples were stained for 1 h at 4°C with 5 μL of phycoerythrin (PE)-conjugated anti-mouse IgG antibody (Sigma-Aldrich). The stained samples were spun at 5000 × g for 5 min, and the cell pellet was resuspended in 500 μL of 1% paraformaldehyde buffer and stored at 4°C overnight. Cytometry samples were resuspended in PBS and analyzed in a flow

A

NO.	Sulbactam MIC	ISAba1-bla$_{ADC-30}$	bla$_{TEM-1}$	bla$_{OXA-23-lke}$	bla$_{OXA-24-like}$	bla$_{OXA-58-like}$
8	64	-	-	+	-	-
55	16	+	-	-	+	-
50	32	+	-	+	-	-
22	64	+	-	+	-	-
1	32	+	-	+	-	-
19	32	+	+	-	-	-
56	32	+	-	-	+	-
42	>64	+	+	+	-	-
23	32	+	+	-	-	-
2	16	+	-	-	+	-
39	>64	+	-	-	-	-
21	>64	+	-	+	-	-
18	64	+	+	-	-	-
44	>64	+	+	+	-	-
33	16	+	-	-	-	-
27	16	+	-	+	-	-
38	64	+	-	+	-	-
4	>64	+	+	+	-	-
25	16	+	+	-	-	-
13	32	+	-	-	-	-
17	64	+	+	-	+	-
53	32	+	-	-	+	-
41	16	+	-	-	-	-
24	64	+	+	+	-	-
54	64	+	+	+	-	-
47	>64	+	+	+	+	-
52	32	+	-	+	-	-
46	16	+	-	+	-	-
14	16	+	+	-	-	-
7	32	+	+	+	-	-

B

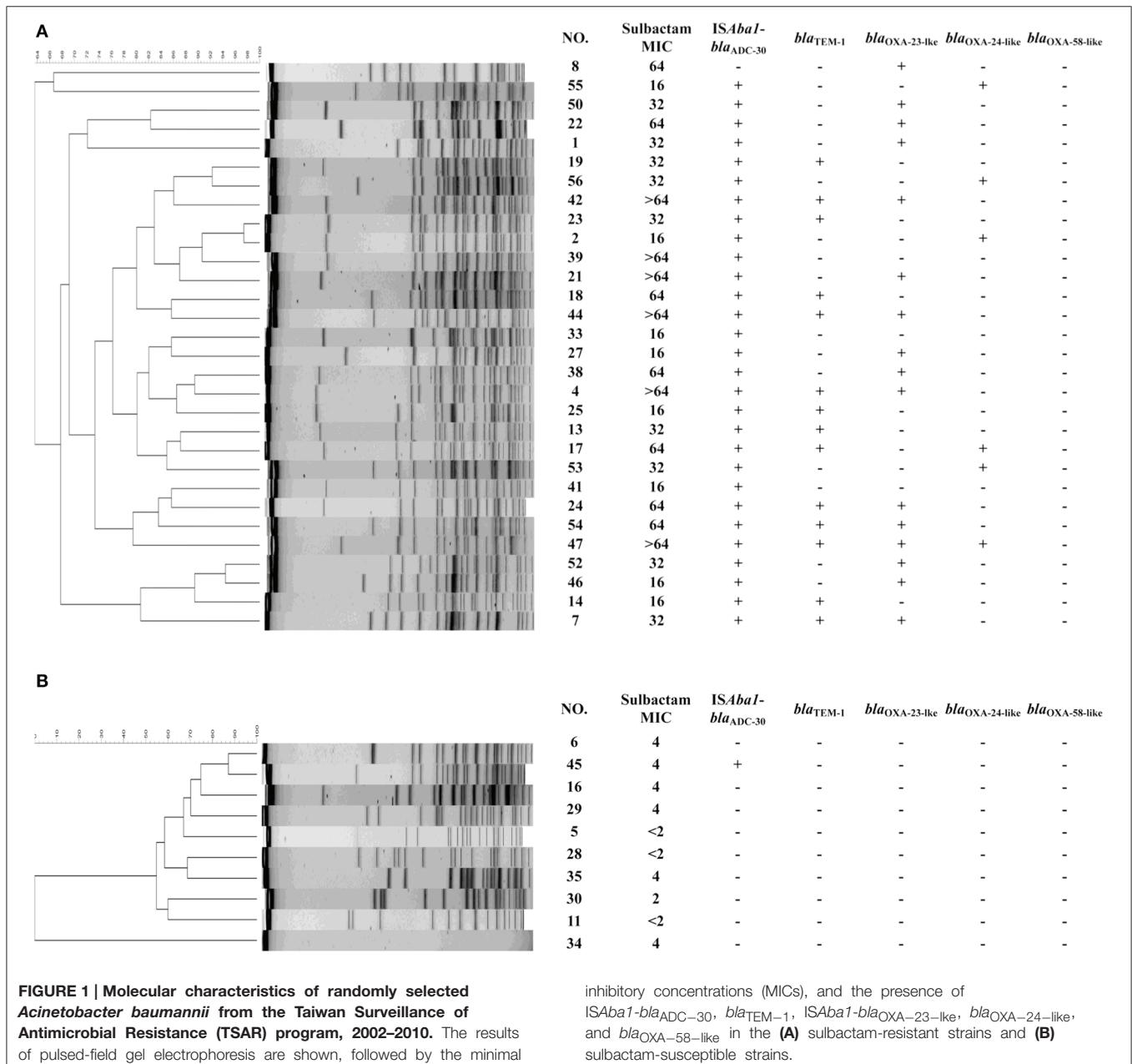| NO.	Sulbactam MIC	ISAba1-bla$_{ADC-30}$	bla$_{TEM-1}$	bla$_{OXA-23-lke}$	bla$_{OXA-24-like}$	bla$_{OXA-58-like}$
6	4	-	-	-	-	-
45	4	+	-	-	-	-
16	4	-	-	-	-	-
29	4	-	-	-	-	-
5	<2	-	-	-	-	-
28	<2	-	-	-	-	-
35	4	-	-	-	-	-
30	2	-	-	-	-	-
11	<2	-	-	-	-	-
34	4	-	-	-	-	-

FIGURE 1 | Molecular characteristics of randomly selected *Acinetobacter baumannii* from the Taiwan Surveillance of Antimicrobial Resistance (TSAR) program, 2002–2010. The results of pulsed-field gel electrophoresis are shown, followed by the minimal inhibitory concentrations (MICs), and the presence of ISAba1-bla$_{ADC-30}$, bla$_{TEM-1}$, ISAba1-bla$_{OXA-23-lke}$, bla$_{OXA-24-like}$, and bla$_{OXA-58-like}$ in the **(A)** sulbactam-resistant strains and **(B)** sulbactam-susceptible strains.

cytometer system with wavelength of 575 nm (FACScanto II, BD Biosciences, San Jose, CA, USA).

Quantitative PCR (qPCR) to Determine the bla_{ADC-30} Gene Copy Number after Challenging with Different Antimicrobials

ATCC15151 (pYMAb2::IS$Aba1$-bla_{ADC-30}) strains at mid-log phase were incubated in Luria-Bertani (LB) broth with different antimicrobial agents (25% of MIC) for 6 h. The bla_{ADC-30} copy number in these bacteria was estimated by qPCR using primers targeting bla_{ADC-30}, and the housekeeping gene, $recA$, was used as an internal control. Each qPCR reaction contained a total volume of 10 μL with 2 ng of genomic DNA as template, 100 nmol/L of each primer, and 1 × SYBR Green® PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) with ROX™ (Kapa Biosystems, Woburn, MA, USA). The relative bla_{ADC-30} copy number in the bacteria treated with different antimicrobial agents was normalized to the number found in the bacteria treated with LB broth without antimicrobial agents. The qPCR conditions included 2 min at 50°C (UNG activation), 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C. At the end, a dissociation stage was added: 15 s at 95°C, 15 s at 60°C, and 15 s at 95°C. All experiments were conducted using the ABI 7500 Fast Real-time PCR system (Applied Biosystems, Inc., Carlsbad, CA, USA) and were performed in triplicate.

Quantitative Reverse Transcription PCR (qRT-PCR) to Assess mRNA Expression after Challenging with Different Antimicrobials

After incubation with different antimicrobial agents, the ADC-30 mRNA levels in ATCC15151 (pYMAb2::IS$Aba1$-bla_{ADC-30}) were compared using qRT-PCR (Chen et al., 2010). Briefly, around 2 μg of RNA was extracted with RNAprotect Bacteria Reagent and an RNeasy mini-kit (Qiagen, Valencia, CA, USA). Residual genomic DNA was removed using RNase-free DNase. The RNA was reverse transcribed into single-stranded cDNA with random hexamers and Moloney Murine Leukemia Virus reverse transcriptase (Epicenter, Madison, WI, USA). The cDNAs were subsequently quantified by real-time PCR amplification with

conditions mentioned above. Expression level results were standardized to the transcription levels of $rpoB$ gene for each strain, but relatively to the culture in LB (2 delta–delta Ct method). Negative controls without reverse transcription were performed to detect DNA contamination in the purified RNA.

ADC-30 Purification

His-tagged ADC-30 (in which the stop codon was deleted, and the proteins were fused with 6 His amino acids) expressed by ATCC15151 (pYMAb2::IS$Aba1$-bla_{ADC-30}) was purified with Ni-NTA Superflow column (Qiagen). Briefly, the bacteria equal to ~10^7 CFU/ml were centrifuged, resuspended in lysis buffer and sonicated. The lysate was diluted in binding buffer (25 mM Tris, 150 mM NaCl, 10 mM imidazole, pH 7.5) and loaded onto the column. The column was washed with five column volumes of wash buffer, and the protein was eluted with five column volumes of elution buffer (25 mM Tris, 150 mM NaCl, 300 mM imidazole). The protein solution was then dialyzed and concentrated by ultrafiltration on a 10 KDa-cutoff Amicon membrane (Millipore). The purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting as greater than 95%.

SDS-PAGE and Western Blotting to Detect Purified β-Lactamases

For SDS-PAGE and Western blotting (Blair et al., 2009), the purified protein was first separated by SDS-PAGE on 12% acrylamide gel using Mini-PROTEAN system (Bio-Rad) and transferred to a nitrocellulose membrane (PerkinElmer, Boston, MA, USA) in transfer buffer (25 mM Tris-HCl, 190 mM glycine, 20% methanol, 0.1% SDS, pH 8.3) at 350 mA for 1 h. The membranes were blocked with 10% non-fat dried milk in Tris-buffered saline with 0.5% Tween-20. After hybridization with anti-His antibodies (Sigma-Aldrich) and peroxidase-conjugated goat anti-mouse secondary antibodies (Millipore, Temecula, CA, USA), the band was visualized with an ECL Western blot kit (PerkinElmer, Boston, MA, USA).

Bioassays to Confirm the Activity of Purified ADC-30 against Ceftazidime and Sulbactam

The first bioassay involved the mixture of 10 μL of purified ADC-30 or extracts of sulbactam- resistant or -susceptible strains with

TABLE 1 | Minimal inhibitory concentrations (μg/ml) of *Acinetobacter baumannii* reference strain and different transformants.

| Strains	Sulbactam	Ceftazidime	Ampicillin	Imipenem	Meropenem	Ticarcillin
ATCC15151	2	8	64	0.25	0.5	16
ATCC15151 (pYMAb2)	2	8	64	0.25	0.5	16
ATCC15151 (pYMAb2::IS$Aba1$-bla_{ADC-30})	32	512	>1024	2	4	128
ATCC15151 (pYMAb2::bla_{ADC-30} Δ P1 [deletion of -35 promoter])	4	32	256	0.25	0.25	32
ATCC15151 (pYMAb2::bla_{ADC-30} ΔP2 [deletion of -10 and -35 promoter])	4	32	64	0.5	0.25	16
ATCC15151 (pYMAb2::IS$Aba1$-bla_{OXA-23})	16	8	>1024	16	16	>1024
ATCC15151 (pYMAb2::$bla_{OXA-24/72}$)	4	8	1024	16	64	512
ATCC15151 (pYMAb2::IS1008–ΔIS$Aba3$-bla_{OXA-58})	8	4	>1024	16	8	>1024
ATCC15151 (pYMAb2:: bla_{TEM-1})	16	8	>1024	0.25	0.25	>1024
ATCC15151 (pYMAb2:: bla_{TEM-1} ΔP [deletion of promoter])	4	8	64	0.25	0.25	32
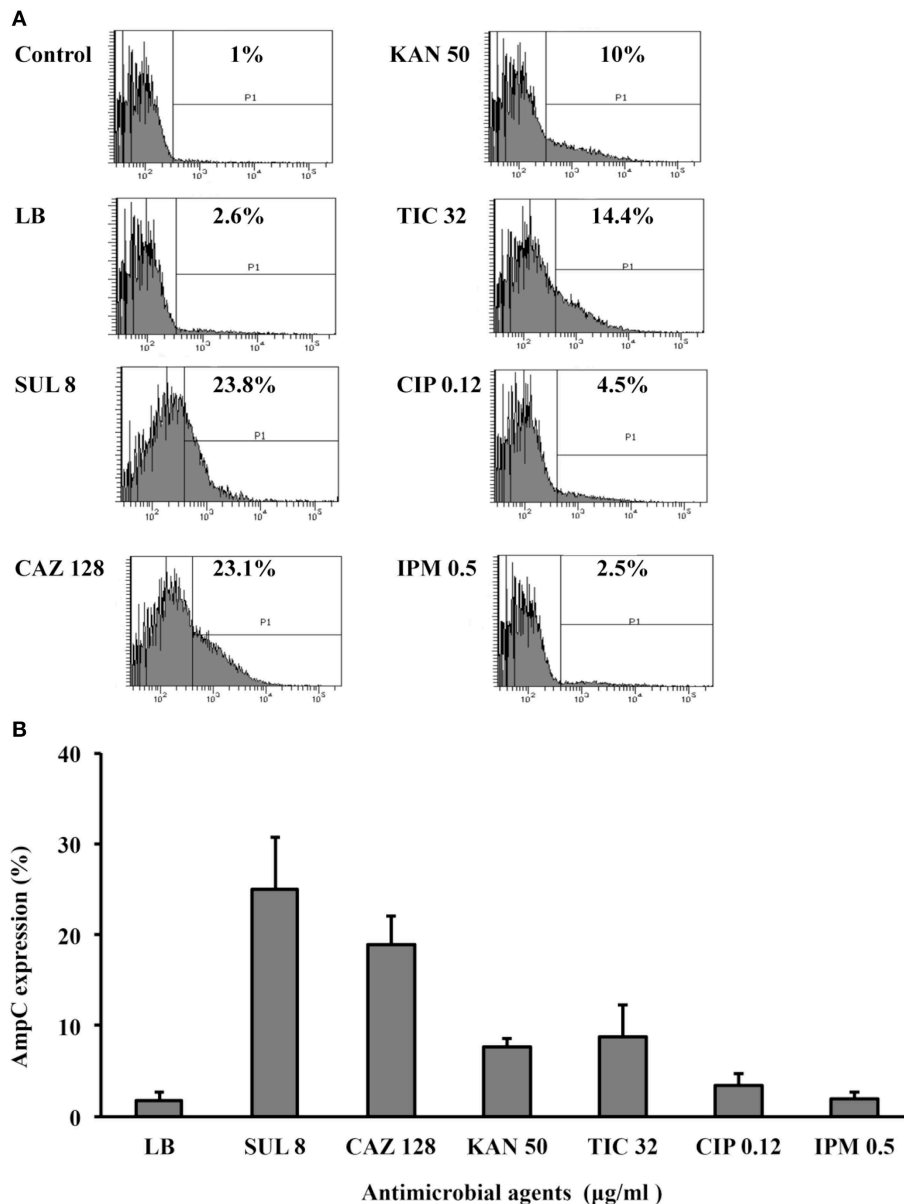
FIGURE 2 | ADC-30 production in response to treatment with sulbactam and other substrates. (A) Flow cytometry showed that the ADC-30 protein expression level increased in response to its substrates (ceftazidime, sulbactam, and ticarcillin) compared with the level observed in bacteria cultured in Luria-Bertani (LB) broth without antimicrobial agents. The tests were performed in triplicate; however, only one of the representative experiments is shown. **(B)** Quantified values of experiments performed in triplicate are shown in the bar graph. LB broth supplemented with kanamycin was used as a positive control because pYMAb2 also carries a kanamycin resistance determinant. CAZ, ceftazidime; CIP, ciprofloxacin; IPM, imipenem; KAN, kanamycin; SUL, sulbactam.

10 µL of ceftazidime for 30 min at 37°C. Each 20-µL mixture was loaded onto a blank disk (Becton Dickinson, Sparks, MD, USA) that was placed in an agar plate containing a lawn of ceftazidime-susceptible *A. baumannii* ATCC 15151. Inhibitory zones were measured after incubating the plates overnight at 37°C.

The second bioassay conducted was similar to the modified Hodge test for carbapenemase detection. A 0.5 McFarland standard suspension of the sulbactam-susceptible *A. baumannii* ATCC 15151 strain was diluted to 1:10 and inoculated on an Mueller-Hinton agar plate for routine disk diffusion test. The 30-µg sulbactam disk was placed in the center of the plate, and the purified ADC-30, TEM-1, or PBS samples were drawn in a straight line out from the edge of the disk. The phenotype was evaluated after an overnight incubation at 37°C.

Results and Discussion

Detection of Selected β-Lactamases in Clinical *A. baumannii* Isolates using a Nationwide Surveillance System

Of the 30 sulbactam-resistant isolates tested, 14 (47%) were positive for $bla_{TEM-1,}$ and 29 (97%) possessed bla_{ADC} with IS*Aba1* upstream (IS*Aba1*-bla_{ADC}). The PFGE shown in **Figure 1** depicts the strain diversity. Based on clonality, 19 isolates belonging to different clones and carrying IS*Aba1*-bla_{ADC} were sent for sequencing. The bla_{ADC} sequences of all 19 strains were consistent with the bla_{ADC-30} sequence. IS*Aba1*-bla_{OXA-23},

$bla_{OXA-24/72}$ and IS*1008*–ΔIS*Aba3*-bla_{OXA-58} were present in16, 6, and 0 isolates, respectively. In 10 sulbactam-susceptible isolates, one possessed IS*Aba1*-bla_{ADC-30}. None of the isolates possessed bla_{TEM-1}. Therefore, in addition to TEM-1(Krizova et al., 2013), IS*Aba1*-bla_{ADC-30} may play a role in providing *A. baumannii* with sulbactam resistance.

The presence of one resistant strain without IS*Aba1*-bla_{ADC} nor bla_{TEM-1} indicates that another mechanism may be involved in sulbactam resistance. Various combinations of resistance mechanisms, including β-lactamase overexpression, the up-regulation of the efflux pump and the inactivation or down-regulation of porin, are often required for the development of
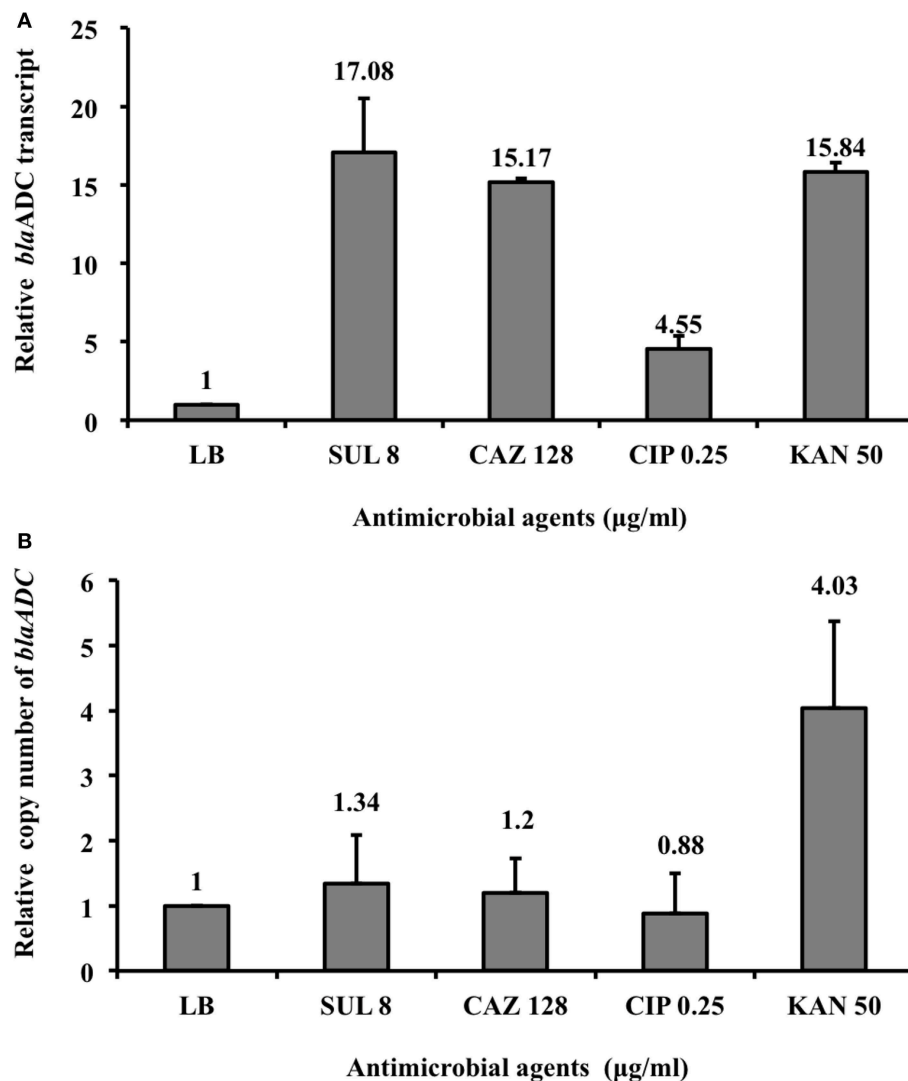

FIGURE 3 | Bla_{ADC-30} mRNA expression level and gene copy number in response to treatment with sulbactam and other antimicrobial agents. (A) The bla_{ADC-30} mRNA expression level increased after treatment with sulbactam and ceftazidime. ATCC15151 (pYMAb2::IS*Aba1*-bla_{ADC-30}) treated with ciprofloxacin or without antimicrobial agents were used as negative controls. **(B)** The bla_{ADC-30} gene copy number did not differ, regardless of the antimicrobial agents added. The mRNA expression level increased in response to kanamycin because of the increased number of plasmids (pYMAb2) carrying the kanamycin-resistant gene. The test was performed in triplicate. CAZ, ceftazidime; CIP, ciprofloxacin; KAN, kanamycin; LB, Luria-Bertani broth; SUL, sulbactam.

β-lactam resistance in *Pseudomonas aeruginosa* and *A. baumannii* (Quale et al., 2006; Peleg et al., 2008; Tiwari et al., 2012; Tiwari and Moganty, 2014), which may explain the presence of IS*Aba1-bla*$_{ADC-30}$ in one susceptible strain. However, the high rate of IS*Aba1-bla*$_{ADC-30}$ in resistant strains and its low rate in susceptible strains (97% vs. 10%, Chi-square test $p < 0.001$) indicate the importance of IS*Aba1-bla*$_{ADC-30}$ for the development of sulbactam resistance.

Contribution of ADC-30 and IS*Aba1* to Sulbactam Resistance

To further confirm the association observed in the epidemiological survey, IS*Aba1-bla*$_{ADC-30}$, *bla*$_{TEM-1}$, IS*Aba1-bla*$_{OXA-23}$, *bla*$_{OXA-24/72}$ (and its promoter), and IS*1008*–ΔIS*Aba3*-*bla*$_{OXA-58}$ were cloned and transformed into a sulbactam-susceptible reference strain, respectively. Shuttle vectors were also transformed, and changes in the MICs were measured (**Table 1**).

ATCC15151 (pYMAb2::IS*Aba1-bla*$_{ADC-30}$) exhibited the highest increases of sulbactam MIC (16-fold). Other β-lactamases, including OXA-23, OXA-72, and OXA-58, contributed to the increase in the sulbactam MICs, although at

FIGURE 4 | Activity of ADC-30 against ceftazidime and sulbactam. (A) ADC-30 remained active against ceftazidime in *Acinetobacter baumannii* after purification. Disks containing ceftazidime and different samples were placed in an agar plate containing a lawn of ceftazidime-susceptible *A. baumannii* ATCC 15151. Crude extract of the ATCC15151 (pYMAb2::IS*Aba1-bla*$_{ADC-30}$) strain and purified ADC-30 (denoted as S1 and S2 in the lower filed) promoted the growth of the *A. baumannii* ATCC 15151 strain against disks loaded with ceftazidime. Controls included N1 (phosphate buffered saline) and N2 [crude extract of ATCC15151 (pYMAb2)], with which the ceftazidime created an inhibitory zone. **(B)** The bioassay resembling modified the Hodge test showed that purified ADC-30 and TEM-1 both promoted *A. baumannii* ATCC 15151 growth against sulbactam. PBS, phosphate buffered saline.

lower levels. IS*Aba1-bla*$_{ADC-30}$ was used in the study due to the high level of sulbactam resistance and high prevalence of IS*Aba1-bla*$_{ADC-30}$, which were comparable to the sulbactam resistance levels and prevalence of *bla*$_{TEM-1}$.

The regulatory mechanism for ADC expression in *Acinetobacter* spp. may be different from the mechanism in many *Enterobacteriaceae* because *Acinetobacter* spp. lack the *AmpR* gene (Jacoby, 2009). The presence of IS*Aba1* upstream of *bla*$_{AmpC}$ is essential for ceftazidime resistance due to the AmpC overexpression (Heritier et al., 2006). To confirm the role of the promoter located within IS*Aba1* in mediating sulbactam resistance, plasmids harboring *bla*$_{ADC-30}$ without upstream -35 (within IS*Aba1*) or -35/-10 promoters were transformed into ATCC15151. The MICs increased by only 2-fold compared with the control (**Table 1**). Therefore, as previous studies have indicated (Heritier et al., 2006), these promoters in the IS*Aba1* are required for ADC-mediated sulbactam resistance. Similarly, deletion of the promoter upstream of *bla*$_{TEM-1}$ decreased the sulbactam MIC.

ADC-30 Production in Response to Treatment with Sulbactam and Other Substrates

The production of β-lactamase usually increases in response to its substrates; therefore, we asked whether the addition of sulbactam increases ADC-30 production. Flow cytometry (**Figure 2**) showed that ADC-30 protein expression increased significantly after the addition of sulbactam or ceftazidime compared with the control. ADC-30 production was also induced by ticarcillin but not by ciprofloxacin or imipenem. The results indicate that the co-selection of sulbactam resistance by other antimicrobial agents occurs.

The qRT-PCR results (**Figure 3**) showed that ADC-30 mRNA expression increased in response to its substrates (ceftazidime and sulbactam) but not in response to other antimicrobial agents (ciprofloxacin). Carbapenemase expression has been related to the high *bla*$_{OXA-58}$ plasmid copy number (Bertini et al., 2007; Chen et al., 2008). Therefore, we asked whether the increased ADC-30 protein and mRNA expression levels in the presence of sulbactam or ceftazidime are attributed to an increase in the *bla*$_{ADC-30}$ copy number. The qPCR results (**Figure 3**) showed no differences in the *bla*$_{ADC-30}$ copy numbers of cells treated with sulbactam or ceftazidime compared with the negative controls. In contrast, the gene copy number increased in response to kanamycin. The results indicate that the increase in ADC-30 was regulated at the RNA level.

How the addition of sulbactam increased ADC-30 protein and mRNA expression level in *A. baumannii* is unknown. The induction of AmpC by β-lactams has been commonly described in *P. aeruginosa* and many *Enterobacteriaceae* (Jacoby, 2009). After the treatment of β-lactams, altered peptidoglycan synthesis leads to increased expression of AmpC through AmpG–AmpR–AmpC pathway (Zeng and Lin, 2013). Clavulanate, another β-lactamase inhibitor with structure similar to β-lactams, also induces the expression of AmpC in many *Enterobacteriaceae* (Drawz and Bonomo, 2010). Although sulbactam may interfere the wall synthesis by interacting with penicillin-binding protein 1 and 3 (Penwell et al., 2015), lack of *AmpR* in *A. baumannii* indicated other mechanisms, rather than AmpG–AmpR–AmpC
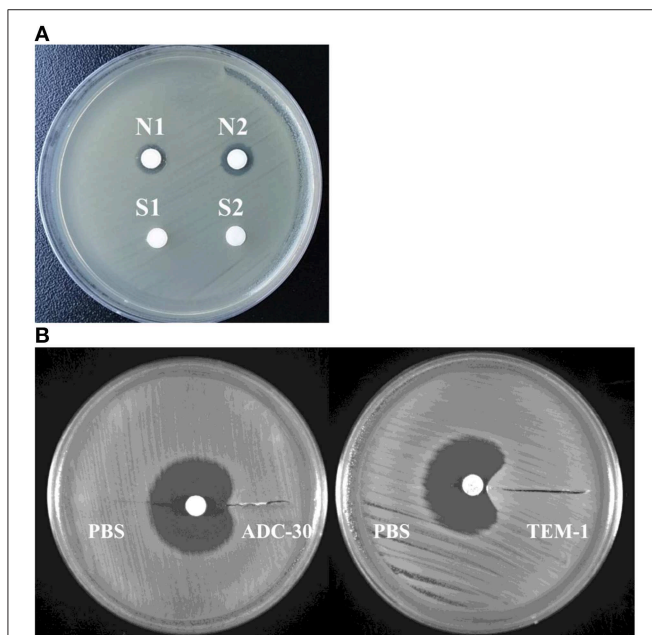
pathway, are responsible. The two-component system has been proposed to be involved in the induction of AmpC and other chromosomally encoded β-lactamases (Jacoby, 2009; Zeng and Lin, 2013). However, the mechanism regarding the induction of ADC in response to sulbactam in *A. baumannii* requires further investigation.

Contribution of Purified ADC-30 to Sulbactam Resistance

ADC-30 was purified (Figure S1), and the addition of purified ADC-30 to the disk decreased the inhibitory zone caused by ceftazidime (**Figure 4A**), confirming the activity of the purified protein. The bioassay resembling the modified Hodge test showed that purified ADC-30 promoted the inward growth of sulbactam-susceptible *A. baumannii* similarly to purified TEM-1 (**Figure 4B**). Therefore, ADC-30 may directly interact with sulbactam and confer sulbactam resistance.

In conclusion, bla_{ADC-30} overexpression contributes to sulbactam resistance in *A. baumannii*, which is prevalent in clinical isolates of different clones in Taiwan over a long period of time. The resistance mechanisms are induced at the mRNA and protein levels by other antimicrobial agents in addition to sulbactam, supporting the cautious use of these antibiotics to avoid the selection of sulbactam-resistant *A. baumannii* isolates.

Ethical Approval

This study was approved by the Institutional Review Board of National Health Research Institutes.

Supplementary Material

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2015.00231/abstract

References

Adnan, S., Paterson, D. L., Lipman, J., and Roberts, J. A. (2013). Ampicillin/sulbactam: its potential use in treating infections in critically ill patients. *Int. J. Antimicrob. Agents* 42, 384–389. doi: 10.1016/j.ijantimicag.2013.07.012

Bertini, A., Poirel, L., Bernabeu, S., Fortini, D., Villa, L., Nordmann, P., et al. (2007). Multicopy bla_{OXA-58} gene as a source of high-level resistance to carbapenems in *Acinetobacter baumannii. Antimicrob. Agents Chemother.* 51, 2324–2328. doi: 10.1128/AAC.01502-06

Blair, J. M., La Ragione, R. M., Woodward, M. J., and Piddock, L. J. (2009). Periplasmic adaptor protein AcrA has a distinct role in the antibiotic resistance and virulence of *Salmonella enterica* serovar Typhimurium. *J. Antimicrob. Chemother.* 64, 965–972. doi: 10.1093/jac/dkp311

Chen, T. L., Chang, W. C., Kuo, S. C., Lee, Y. T., Chen, C. P., Siu, L. K., et al. (2010). Contribution of a plasmid-borne bla_{OXA-58} gene with its hybrid promoter provided by IS*1006* and an IS*Aba3*-like element to beta-lactam resistance in *Acinetobacter* genomic species 13TU. *Antimicrob. Agents Chemother.* 54, 3107–3112. doi: 10.1128/AAC.00128-10

Chen, T. L., Siu, L. K., Wu, R. C., Shaio, M. F., Huang, L. Y., Fung, C. P., et al. (2007). Comparison of one-tube multiplex PCR, automated ribotyping and intergenic spacer (ITS) sequencing for rapid identification of *Acinetobacter baumannii. Clin. Microbiol. Infect.* 13, 801–806. doi: 10.1111/j.1469-0691.2007.01744.x

Chen, T. L., Wu, R. C., Shaio, M. F., Fung, C. P., and Cho, W. L. (2008). Acquisition of a plasmid-borne bla_{OXA-58} gene with an upstream IS*1008* insertion conferring a high level of carbapenem resistance to *Acinetobacter baumannii. Antimicrob. Agents Chemother.* 52, 2573–2580. doi: 10.1128/AAC.00393-08

Clinical and Laboratory Standards Institute. (2012). *Performance Standards for Antimicrobial Susceptibility Testing. Twenty-Second Informational Supplement. CLSI Document M100-S22.* Wayne, PA: CLSI.

Corbella, X., Ariza, J., Ardanuy, C., Vuelta, M., Tubau, F., Sora, M., et al. (1998). Efficacy of sulbactam alone and in combination with ampicillin in nosocomial infections caused by multiresistant *Acinetobacter baumannii. J. Antimicrob. Chemother.* 42, 793–802. doi: 10.1093/jac/42.6.793

Drawz, S. M., and Bonomo, R. A. (2010). Three decades of beta-lactamase inhibitors. *Clin. Microbiol. Rev.* 23, 160–201. doi: 10.1128/CMR.00037-09

Gales, A. C., Jones, R. N., and Sader, H. S. (2011). Contemporary activity of colistin and polymyxin B against a worldwide collection of Gram-negative pathogens: results from the SENTRY Antimicrobial Surveillance Program (2006-09). *J. Antimicrob. Chemother.* 66, 2070–2074. doi: 10.1093/jac/dkr239

Heritier, C., Poirel, L., and Nordmann, P. (2006). Cephalosporinase overexpression resulting from insertion of IS*Aba1* in *Acinetobacter baumannii. Clin. Microbiol. Infect.* 12, 123–130. doi: 10.1111/j.1469-0691.2005.01320.x

Jacoby, G. A. (2009). AmpC beta-lactamases. *Clin. Microbiol. Rev.* 22, 161–182. doi: 10.1128/CMR.00036-08

Karageorgopoulos, D. E., and Falagas, M. E. (2008). Current control and treatment of multidrug-resistant *Acinetobacter baumannii* infections. *Lancet Infect. Dis.* 8, 751–762. doi: 10.1016/S1473-3099(08)70279-2

Ko, W. C., Lee, H. C., Chiang, S. R., Yan, J. J., Wu, J. J., Lu, C. L., et al. (2004). *In vitro* and *in vivo* activity of meropenem and sulbactam against a multidrug-resistant *Acinetobacter baumannii* strain. *J. Antimicrob. Chemother.* 53, 393–395. doi: 10.1093/jac/dkh080

Krizova, L., Poirel, L., Nordmann, P., and Nemec, A. (2013). TEM-1 beta-lactamase as a source of resistance to sulbactam in clinical strains of *Acinetobacter baumannii. J. Antimicrob. Chemother.* 68, 2786–2791. doi: 10.1093/jac/dkt275

Kuo, S. C., Chang, S. C., Wang, H. Y., Lai, J. F., Chen, P. C., Shiau, Y. R., et al. (2012). Emergence of extensively drug-resistant *Acinetobacter baumannii* complex over 10 years: nationwide data from the Taiwan Surveillance of Antimicrobial Resistance (TSAR) program. *BMC Infect. Dis.* 12:200. doi: 10.1186/1471-2334-12-200

Kuo, S. C., Yang, S. P., Lee, Y. T., Chuang, H. C., Chen, C. P., Chang, C. L., et al. (2013). Dissemination of imipenem-resistant *Acinetobacter baumannii* with new plasmid-borne bla_{OXA-72} in Taiwan. *BMC Infect. Dis.* 13:319. doi: 10.1186/1471-2334-13-319

Moe, G. R., Tan, S., and Granoff, D. M. (1999). Differences in surface expression of NspA among *Neisseria meningitidis* group B strains. *Infect. Immun.* 67, 5664–5675.

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

Penwell, W. A., Shapiro, A. B., Giacobbe, R. A., Gu, R. F., Gao, N., Thresher, J., et al. (2015). The molecular mechanisms of sulbactam antibacterial activity and resistance determinants in *Acinetobacter baumannii. Antimicrob. Agents Chemother.* 59, 1680–1689. doi: 10.1128/AAC.04808-14

Quale, J., Bratu, S., Gupta, J., and Landman, D. (2006). Interplay of efflux system, ampC, and oprD expression in carbapenem resistance of *Pseudomonas aeruginosa* clinical isolates. *Antimicrob. Agents Chemother.* 50, 1633–1641. doi: 10.1128/AAC.50.5.1633-1641.2006

Song, J. Y., Kee, S. Y., Hwang, I. S., Seo, Y. B., Jeong, H. W., Kim, W. J., et al. (2007). *In vitro* activities of carbapenem/sulbactam combination, colistin, colistin/rifampicin combination and tigecycline against carbapenem-resistant *Acinetobacter baumannii. J. Antimicrob. Chemother.* 60, 317–322. doi: 10.1093/jac/dkm136

Tiwari, V., Kapil, A., and Moganty, R. R. (2012). Carbapenem-hydrolyzing oxacillinase in high resistant strains of *Acinetobacter baumannii* isolated from India. *Microb. Pathog.* 53, 81–86. doi: 10.1016/j.micpath.2012.05.004

Tiwari, V., Khokar, M. K., Tiwari, M., Barala, S., and Kumar, M. (2014). Antibacterial activity of polyvinyl pyrrolidone capped silver nanoparticles on the carbapenem resistant strain of *Acinetobacter baumannii. J. Nanomed. Nanotechnol.* 5:1000246. doi: 10.4172/2157-7439.1000246

Tiwari, V., and Moganty, R. R. (2014). Conformational stability of OXA-51 beta-lactamase explains its role in carbapenem resistance of *Acinetobacter baumannii. J. Biomol. Struct. Dyn.* 32, 1406–1420. doi: 10.1080/07391102.2013.819789

Wolff, M., Joly-Guillou, M. L., Farinotti, R., and Carbon, C. (1999). *In vivo* efficacies of combinations of beta-lactams, beta-lactamase inhibitors, and rifampin against *Acinetobacter baumannii* in a mouse pneumonia model. *Antimicrob. Agents Chemother.* 43, 1406–1411.

Zeng, X., and Lin, J. (2013). Beta-lactamase induction and cell wall metabolism in Gram-negative bacteria. *Front. Microbiol.* 4:128. doi: 10.3389/fmicb.2013.00128