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Genetic Basis of Multidrug Resistance in *Acinetobacter* Clinical Isolates in Taiwan▽

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Multidrug-resistant (MDR) *Acinetobacter* spp. have emerged as a threat to public health. We investigated the various genes involved in resistance to fluoroquinolones, aminoglycosides, cephalosporins, and carbapenems in 75 clinical *Acinetobacter* isolates from a Taiwanese hospital. All isolates were tested for the *gyrA* mutations, the presence of integrons, bla_{AmpC}, and carbapenem resistance genes. The Ser83Leu mutation in GyrA accounted for fluoroquinolone resistance. The presence of integrons containing aminoglycoside-modifying enzymes was associated with resistance to gentamicin and tobramycin but not with resistance to amikacin. The presence of an IS*Aba1* element upstream of bla_{AmpC} was correlated with cephalosporin resistance. Although most *Acinetobacter baumannii* isolates with IS*Aba1*-$bla_{OXA-51-like}$ were resistant to carbapenems, several isolates remained susceptible to carbapenems. Transformation by the introduction of IS*Aba1*-bla_{OXA-23} or IS*Aba1*-bla_{OXA-66} into *A. baumannii* ATCC 15151 (CIP 70.10), resulting in the overexpression of OXA-23 or OXA-66, respectively, suggested the role of the IS*Aba1* element as a strong promoter. The two transformants showed significantly increased resistance to piperacillin-tazobactam, imipenem, and meropenem. The cefepime resistance conferred by IS*Aba1*-bla_{OXA-23} and the impact of IS*Aba1*-bla_{OXA-66} on carbapenem resistance in *A. baumannii* are reported here for the first time. Continuous surveillance of antibiotic resistance genes in MDR *Acinetobacter* spp. and elucidation of their antibiotic resistance mechanisms are crucial for the development of therapy regimens and for the prevention of further dissemination of these antibiotic resistance genes.

In the past 2 decades, *Acinetobacter* spp. have become important opportunistic pathogens responsible for nosocomial infections, especially among patients in intensive care units (ICUs) (27). Infections with nosocomial *Acinetobacter* spp. have created a challenge for concordant therapy due to their acquisition of multidrug-resistant (MDR) phenotypes, such as resistance to fluoroquinolones, aminoglycosides, cephalosporins, and carbapenems (7).

Fluoroquinolone-resistant *Acinetobacter* spp. have emerged rapidly following an increase in the consumption of fluoroquinolones (primarily ciprofloxacin) (22). A major change from Ser83 to Leu83 (Ser83Leu) in the quinolone resistance-determining region (QRDR) of DNA gyrase subunit A (GyrA) is highly correlated with resistance to ciprofloxacin in *Acinetobacter baumannii* (36, 40). In addition, MDR *Acinetobacter* spp. frequently contain integrons, which provide bacteria with a gene capture system perfectly adapted to circumvent the challenges of multiple-antibiotic treatment regimens (24). Currently, a total of five classes of mobile integrons are known to play roles in the dissemination of antibiotic resistance genes (24). Class 1 integrons are the predominant class of integrons found in *A. baumannii* and contain the vast majority of gene cassettes encoding various aminoglycoside-modifying enzymes (13). The presence of such integrons may be associated with aminoglycoside resistance in *Acinetobacter* spp.

Ambler class C β-lactamases, known as AmpC cephalosporinases, are chromosomally inherited in *A. baumannii*. The major regulator of bla_{AmpC} is an upstream insertion sequence (IS) element, IS*Aba1* (5, 35). The presence of IS*Aba1*-bla_{AmpC} in *A. baumannii* is highly correlated with overexpression of bla_{AmpC} and resistance to extended-spectrum cephalosporins but not to carbapenems (5, 11, 35). Carbapenems are the broadest-spectrum β-lactams and are considered a "last resort" for serious infections with Gram-negative bacteria (21). However, carbapenem-resistant *Acinetobacter* spp. have emerged worldwide and are often associated with the presence of Ambler class B metallo-β-lactamases (MBLs) and carbapenem-hydrolyzing class D β-lactamases (CHDLs) (28). Two main MBL genes (bla_{IMP} and bla_{VIM}), and four main CHDL genes (the bla_{OXA-23}, $bla_{OXA-24-like}$, $bla_{OXA-51-like}$, and bla_{OXA-58} genes) have been identified in carbapenem-resistant *Acinetobacter* spp. (30, 33). The $bla_{OXA-51-like}$ gene is chromosomally intrinsic only to *A. baumannii* (9). Furthermore, the IS*Aba1* element is often found upstream of bla_{OXA-23} and $bla_{OXA-51-like}$, and it may provide a promoter that allows the overexpression of the two genes (12, 26).

In this study, we investigate the various genes of *Acinetobacter* spp. from a Taiwanese hospital that are involved in fluoroquinolone, aminoglycoside, cephalosporin, and carbapenem resistance. The roles of IS*Aba1*-bla_{OXA-23} and IS*Aba1*-bla_{OXA-66} in *A. baumannii* are also demonstrated.

MATERIALS AND METHODS

Bacterial strains and plasmids. A total of 75 nonduplicated *Acinetobacter* clinical isolates were collected from the National Taiwan University Hospital (Taipei, Taiwan) in 2006 and were identified by the API 20NE system. Geno-

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TABLE 1. Oligonucleotide primers used in this study

Name	Nucleotide sequence (5′–3′)	Location	Reference
Ab-GF	ACAAGAAATCTGCTCGT	*gyrA*	36
Ab-GR	CGAAGTTACCCTGACCATC	*gyrA*	36
5′-CS	GGCATCCAAGCAGCAAG	Integron	18
3′-CS	AAGCAGACTTGACCTGA	Integron	18
ACI10	GCTGAACGCGATAAACTTC	IS*Aba1* of bla_{AmpC}	5
ACI2	TAGTACTGCTATTTACGGCT	bla_{AmpC}	5
IMP-A	GAAGGYGTTTATGTTCATAC	bla_{IMP}	29
IMP-B	GTAMGTTTCAAGAGTGATGC	bla_{IMP}	29
VIM2004A	GTTTGGTCGCATATCGCAAC	bla_{VIM}	29
VIM2004B	AATGCGCAGCACCAGGATAG	bla_{VIM}	29
OXA23-F	GATCGGATTGGAGAACCAGA	bla_{OXA-23}	40
OXA23-R	ATTTCTGACCGCATTTCCAT	bla_{OXA-23}	40
OXA24-F	GGTTAGTTGGCCCCCTTAAA	$bla_{OXA-24-like}$	40
OXA24-R	AGTTGAGCGAAAAGGGGATT	$bla_{OXA-24-like}$	40
OXA51-F	TAATGCTTTGATCGGCCTTG	$bla_{OXA-51-like}$	40
OXA51-R	TGGATTGCACTTCATCTTGG	$bla_{OXA-51-like}$	40
OXA-58-F	AAGTATTGGGGCTTGTGCTG	bla_{OXA-58}	40
OXA-58-R	CCCCTCTGCGCTCTACATAC	bla_{OXA-58}	40
OXA-58A	CGATCAGAATGTTCAAGCGC	bla_{OXA-58}	31
OXA-58B	ACGATTCTCCCCTCTGCGC	bla_{OXA-58}	31
ISAba3B	CGTTTACCCCAAACATAAGC	*tnpA* of IS*Aba3* (but not in IS*Aba3*-like)	31
ISAba3C	AGCAATATCTCGTATACCGC	*tnpA* of *ISAba3*-like and IS*Aba3*	31
ISAba1-F	GGATCCCTCTGTACACGAYAAATTTC	IS*Aba1*	This study
OXA23-R1	GAATTCTTAAATAATATTCAGCTGTTTTAATG	bla_{OXA-23}	This study
OXA51-R1	GAATTCCTATAAAATACCTAATTGTTCTAAAC	$bla_{OXA-51-like}$	This study

species were identified according to the 16S–23S rRNA gene intergenic spacer (ITS) region as described previously (20). *Escherichia coli* TOP10 and *A. baumannii* ATCC 15151 (CIP 70.10) were used as hosts for the cloning and expression experiments, respectively. Plasmid pCR2.1-TOPO (Invitrogen) was used as the cloning vector, and shuttle plasmid pAT801, with a PvuII fragment harboring the *oriC* of *Acinetobacter* from pW1277 (15) in pUc18, was obtained from P. Courvalin of Institut Pasteur and was used as the expression vector. *E. coli* and *A. baumannii* were cultured on Luria-Bertani broth (LB) and tryptic soy broth (TSB) plates, respectively, at 37°C for 20 h.

Antibiotic susceptibility testing. Antibiotic susceptibility testing was performed using the Vitek 2 automatic system (bioMérieux), with the AST-GN09 card for the identification of Gram-negative bacilli. The MIC of imipenem was further determined by Etest (AB Biodisk, Solna, Sweden). The criteria used were in accordance with the guidelines established by the Clinical and Laboratory Standards Institute (CLSI) (4).

Detection of antibiotic resistance genes. Genomic DNA was extracted as described previously (20). All primers used in this study are listed in Table 1. The QRDR of *gyrA* was amplified and sequenced by procedures similar to those used in our previous study (36). The *gyrA* QRDR was defined as *gyrA* nucleotide positions 241 to 243 in *Acinetobacter* spp., and it is equivalent to Ser83 of *E. coli*. Integrons were amplified and sequenced with primers derived from the 5′ and 3′ conserved segments (18), and additional internal primers were designed to ensure complete identification. The carbapenem resistance genes, including bla_{IMP}, bla_{VIM}, bla_{OXA-23}, $bla_{OXA-24-like}$, $bla_{OXA-51-like}$, and bla_{OXA-58}, were detected by using primers and PCR conditions described previously (29, 41). The presence of the IS*Aba1* element was detected by PCR in order to determine the proximity of this element to bla_{OXA-23}, $bla_{OXA-51-like}$, and bla_{AmpC} (5, 12, 39). The insertion sequence preceding the bla_{OXA-58} gene was detected by PCR using a reverse primer of the *bla* gene and a forward primer of the IS (IS*Aba1*, IS*Aba2*, or IS*Aba3*) (31).

Cloning of IS*Aba1*-bla_{OXA-23} and IS*Aba1*-bla_{OXA-66}. For cloning and verification of the nucleotide sequences of IS*Aba1*-bla_{OXA-23} and IS*Aba1*-$bla_{OXA-51-like}$, primers ISAba1-F and OXA23-R1 were used to amplify IS*Aba1*-bla_{OXA-23} from strain 6AB15, and primers ISAba1-F and OXA51-R1 were used to amplify IS*Aba1*-$bla_{OXA-51-like}$ from strain 6AB11. The PCR conditions for IS*Aba1*-bla_{OXA-23} and IS*Aba1*-$bla_{OXA-51-like}$ were as follows: preheating at 95°C for 2 min; 35 cycles consisting of 95°C for 10 s, 50°C for 10 s, and 72°C for 130 s; and a final extension at 72°C for 60 s. The two PCR products were separately ligated into the pCR2.1-TOPO vector, generating pCR2.1-OXA23 and pCR2.1-OXA51. The inserts of pCR2.1-OXA23 and pCR2.1-OXA51 were sequenced with an Applied Biosystems sequencer (ABI 3730). The nucleotide and deduced protein sequences were analyzed with software available on the website of the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov).

Expression of bla_{OXA-23} and bla_{OXA-66}. The sequence analysis identified $bla_{OXA-51-like}$ from 6AB11 as bla_{OXA-66}. Fragments containing IS*Aba1*-bla_{OXA-23} and IS*Aba1*-bla_{OXA-66} were obtained from pCR2.1-OXA23 and pCR2.1-OXA51, respectively, by digestion with BamHI/EcoRI. BamHI/EcoRI-restricted IS*Aba1*-bla_{OXA-23} and BamHI/EcoRI-restricted IS*Aba1*-bla_{OXA-66} were ligated into BamHI/EcoRI-restricted pAT801, generating pOXA23 and pOXA66, respectively. pOXA23 and pOXA66 were separately transformed into *A. baumannii* ATCC 15151 by electroporation. The transformants were selected on LB plates containing 100 mg/liter ampicillin.

SDS-PAGE analysis. Bacterial cells were grown overnight at 37°C in LB, harvested by centrifugation at 8,000 × *g* for 5 min, and washed once with iced phosphate-buffered saline (PBS). The cells were resuspended in fresh PBS with 1 mM dithiothreitol (DTT) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and were disrupted by sonication. The crude cell extracts were clarified by centrifugation at 12,000 × *g* for 10 min at 4°C, and the supernatants were collected. The protein concentration of the crude cell extract was determined using a commercial bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL) with bovine serum albumin (BSA) (0.05 to 2 mg/ml) as the standard. The crude cell extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 4- to-20% gradient polyacrylamide gel, and protein patterns were detected by staining with Coomassie blue.

Statistical analysis. Statistical analysis was performed using SPSS, version 10.0.7. Pearson's correlation coefficient was used to determine the relationships between the MIC distributions of antibiotic agents and antibiotic resistance genes. An r value of >0.7 (or <-0.7) and a P value of <0.05 were considered statistically significant.

Nucleotide sequence accession numbers. The nucleotide sequences of IS*Aba1*-bla_{OXA-23} and IS*Aba1*-bla_{OXA-66}, reported in this paper, have been submitted to NCBI GenBank under accession numbers GQ849192 and GQ849191, respectively.

RESULTS

The 75 *Acinetobacter* clinical isolates were assigned to three distinct *Acinetobacter* genospecies based on their ITS sequences: *A. baumannii* ($n = 53$), *Acinetobacter* genospecies 13TU ($n = 20$), and *Acinetobacter* genospecies 3 ($n = 2$). The antibiotic

TABLE 2. Antibiotic susceptibilities of 75 *Acinetobacter* clinical isolates

Antibiotic and MIC (mg/liter)[a]	No. of isolates[b]		
	A. baumannii (n = 53)	*Acinetobacter* genospecies 13TU (n = 20)	*Acinetobacter* genospecies 3 (n = 2)
Ciprofloxacin			
≥4 (R)	45 (44)[a]	2 (2)	1 (1)
2 (I)			
Levofloxacin			
≥8 (R)	24 (24)	1 (2)	1 (1)
4 (I)	19 (19)		
Amikacin			
≥64 (R)	15 (15)[b]	2 (2)	
32 (I)	2 (0)		
Gentamicin			
≥16 (R)	43 (40)	2 (1)	
8 (I)		4 (2)	
Tobramycin			
≥16 (R)	40 (39)	1 (1)	
8 (I)	2 (0)	1 (1)	
Ceftazidime			
≥32 (R)	44 (44)[c]	5 (1)	
16 (I)	1 (0)	2 (1)	1 (0)
Cefepime			
≥32 (R)	34 (34)	6 (2)	1 (0)
16 (I)	8 (8)	1 (0)	
Imipenem			
≥16 (R)	19 (17)[d]	2	
8 (I)	5 (4)	2	
Meropenem			
≥16 (R)	20 (18)	2	
8 (I)	4 (3)	1	

[a] MICs followed by "(R)" indicate resistance; MICs followed by "(I)" indicate intermediate resistance.

[b] The numbers of isolates with the following genetic features are given in parentheses: GyrA mutations (for fluoroquinolones [ciprofloxacin and levofloxacin]), integrons (for aminoglycosides [amikacin, gentamicin, and tobramycin]), IS*Aba1*-bla_{AmpC} structures (for cephalosporins [ceftazidime and cefepime]), and IS*Aba1*-$bla_{\text{OXA-51-like}}$ structures (for carbapenems [imipenem and meropenem]).

TABLE 3. Antibiotic resistance genes of 75 *Acinetobacter* clinical isolates

Antibiotic resistance gene(s)	No. of isolates		
	A. baumannii (n = 53)	*Acinetobacter* genospecies 13TU (n = 20)	*Acinetobacter* genospecies 3 (n = 2)
QRDR of GyrA			
Ser83 (TCA)	9	18	1
Ser83Leu (TTA)	44	1	1
Ser83Phe (TTT)		1	
Integron			
Not present	13	15	2
aacA4-catB8-aadA1	35		
aacC1-orfX-orfX′-aadA1	3		
arr-3-aacA4		2	
arr-3-aacA4 + $bla_{\text{VIM-11}}$		1	
dhfrXII-orfF-aadA2	2		
$bla_{\text{IMP-1}}$-*aac(6′)-II-aadA4*		2	
AmpC			
Not present	1	18	2
bla_{AmpC}	8		
IS*Aba1*-bla_{AmpC}	44	2	
CHDLs and MBLs			
Not present		15	2
IS*Aba1*-$bla_{\text{OXA-23}}$ + IS*Aba3*-$bla_{\text{OXA-58}}$		1	
IS*Aba3*-$bla_{\text{OXA-58}}$ only		1	
IS*Aba3*-$bla_{\text{OXA-58}}$ + $bla_{\text{IMP-1}}$		2	
IS*Aba3*-$bla_{\text{OXA-58}}$ + $bla_{\text{VIM-11}}$		1	
IS*Aba1*-$bla_{\text{OXA-23}}$ + $bla_{\text{OXA-51-like}}$	1		
$bla_{\text{OXA-24-like}}$ + $bla_{\text{OXA-51-like}}$	1		
$bla_{\text{OXA-51-like}}$ only	27		
IS*Aba1*-$bla_{\text{OXA-51-like}}$ only	24		

susceptibilities and corresponding antibiotic resistance genes of these *Acinetobacter* clinical isolates are shown in Tables 2 and 3, respectively.

Fluoroquinolones. Eighty-five percent of *A. baumannii* isolates (45/53) were resistant to fluoroquinolones, and all of these contained a Ser83Leu mutation in GyrA, except for one ciprofloxacin-resistant isolate with Ser83 in GyrA that was susceptible to levofloxacin. Only 55% (24/44) of the *A. baumannii* isolates described above with a Ser83Leu mutation in GyrA were also resistant to levofloxacin, and 43% (19/44) were intermediate to levofloxacin. Most of the *Acinetobacter* genospecies 13TU isolates (90% [18/20]) were susceptible to ciprofloxacin, with Ser83 in GyrA. Two *Acinetobacter* genospecies 13TU isolates were resistant to ciprofloxacin; one had a Ser83Leu mutation and the other had a Ser83Phe mutation in GyrA, and the isolate with Ser83Leu in GyrA was also resistant to levofloxacin. Of the two *Acinetobacter* genospecies 3 isolates, the one with Ser83Leu in GyrA was resistant to ciprofloxacin and levofloxacin. The GyrA mutations of *Acinetobacter* spp. were significantly correlated with susceptibility to ciprofloxacin (r, 0.972; P, <0.001) and levofloxacin (r, 0.847; P, <0.001).

Aminoglycosides. Among the 75 *Acinetobacter* isolates, six distinct integrons were found, and their gene cassettes contained various aminoglycoside-modifying genes, including *aacA4*, *aacC1*, *aac(6′)-II*, *aadA1*, *aadA2*, and *aadA4*. Three integrons were detected in *A. baumannii*, including *aacA4-catB8-aadA1* (2,381 bp), *aacC1-orfX-orfX′-aadA1* (2,542 bp), and *dhrfXII-orfF-aadA2* (1,873 bp), while three other integrons, including *arr-3-aacA4* (1,395 bp), $bla_{\text{VIM-11}}$ (1,062 bp), and $bla_{\text{IMP-1}}$-*aac(6′)-II-aadA4* (2,507 bp), were detected only in *Acinetobacter* genospecies 13TU. Twenty-eight percent (15/53) of *A. baumannii* isolates were resistant to amikacin and contained integrons. Furthermore, 81% (43/53) and 75% (40/53) of the *A. baumannii* isolates were resistant to gentamicin and tobramycin, respectively, and most of them contained integrons. In *Acinetobacter* genospecies 13TU, five isolates contained integrons, but some of them remained susceptible to aminoglycosides. No integrons were detected in the two *Acinetobacter* genospecies 3 isolates, and they were also susceptible to the three aminoglycosides analyzed. Consequently, the presence of integrons in *Acinetobacter* spp. was significantly correlated with susceptibility to

gentamicin (r, 0.8; P, <0.001) and tobramycin (r, 0.841; P, <0.001) but not to amikacin (r, 0.403; P, <0.001).

Cephalosporins. Eighty-three percent (44/53) of *A. baumannii* isolates were resistant to ceftazidime, and all of them contained the IS*Aba1*-bla_{AmpC} structure. The eight *A. baumannii* isolates lacking IS*Aba1* upstream of bla_{AmpC} were susceptible to ceftazidime, and the sole isolate intermediate to ceftazidime did not contain bla_{AmpC}. Seventy-nine percent (42/53) of *A. baumannii* isolates were resistant to cefepime, and they contained the IS*Aba1*-bla_{AmpC} structure. In *Acinetobacter* genospecies 13TU, seven isolates were resistant or intermediate to ceftazidime, and two of these contained the IS*Aba1*-bla_{AmpC} structure. Furthermore, these isolates were also not susceptible to cefepime and one ceftazidime-intermediate isolate harboring the IS*Aba1*-bla_{AmpC} structure showed resistance to cefepime. In addition, one *Acinetobacter* genospecies 3 isolates without bla_{AmpC} was intermediate to ceftazidime and resistant to cefepime. The presence of IS*Aba1* upstream of bla_{AmpC} in *Acinetobacter* spp. was significantly correlated with susceptibility to ceftazidime (r, 0.861; P, <0.001) and cefepime (r, 0.725; P, <0.001).

Carbapenems. Forty-five percent (24/53) of *A. baumannii* isolates were resistant or intermediate to imipenem and meropenem; most (88% [21/24]) of them contained an IS*Aba1*-$bla_{\text{OXA-51-like}}$ structure. Among the remaining isolates without IS*Aba1* upstream of $bla_{\text{OXA-51-like}}$, one isolate containing IS*Aba1*-$bla_{\text{OXA-23}}$ and one containing $bla_{\text{OXA-24-like}}$ were resistant to carbapenems, while one isolate, intermediate to carbapenems, had no detectable carbapenemase genes. The presence of IS*Aba1* upstream of $bla_{\text{OXA-51-like}}$ in *A. baumannii* was highly correlated with susceptibility to imipenem (r, 0.753; P, <0.001) and meropenem (r, 0.764; P, <0.001). In addition, among the five *Acinetobacter* genospecies 13TU isolates harboring IS*Aba3*-$bla_{\text{OXA-58}}$, one isolate containing only IS*Aba3*-$bla_{\text{OXA-58}}$ was susceptible to carbapenems, while of the other four isolates, containing additional carbapenemase genes, two isolates with $bla_{\text{IMP-1}}$ were resistant to carbapenems, one isolate with IS*Aba1*-$bla_{\text{OXA-23}}$ was intermediate to carbapenems, and one isolate with $bla_{\text{VIM-11}}$ was intermediate to imipenem but susceptible to meropenem. Furthermore, all of the five *Acinetobacter* genospecies 13TU isolates harboring IS*Aba3*-$bla_{\text{OXA-58}}$ were also resistant to ceftazidime and cefepime, and only one of them, harboring $bla_{\text{IMP-1}}$, contained IS*Aba1*-bla_{AmpC}. A total of 15 *Acinetobacter* genospecies 13TU and 2 *Acinetobacter* genospecies 3 isolates harbored no carbapenem resistance genes that we detected, and all of them were susceptible to carbapenems.

Cloning of IS*Aba1*-$bla_{\text{OXA-23}}$ and IS*Aba1*-$bla_{\text{OXA-66}}$. DNA sequence analysis indicated that the insert of pCR2.1-OXA23, with a 2,036-bp fragment size, was 99% identical to IS*Aba1*-$bla_{\text{OXA-23}}$ in *A. baumannii* (GenBank accession number EF127491) and the insert of pCR2.1-OXA51 revealed a 2,012-bp fragment 100% identical to IS*Aba1*-$bla_{\text{OXA-66}}$ in *A. baumannii* (GenBank accession number DQ923479).

Expression of $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-66}}$ in *A. baumannii* ATCC 15151. The crude cell extracts of two transformants, *A. baumannii* ATCC 15151(pOXA23) and *A. baumannii* ATCC 15151(pOXA66), were analyzed by SDS-PAGE (Fig. 1). The protein patterns of clinical isolates 6AB11 and 6AB15 (Fig. 1, lanes 1 and 2) were significantly different from that of *A. baumannii* ATCC 15151 (lane 3), while the protein pattern of the *A. baumannii* transformant ATCC 15151(pAT801) (lane 4) was identical to that of *A. baumannii* ATCC 15151. Furthermore, the protein patterns of the two transformants *A. baumannii* ATCC 15151(pOXA23) and *A. baumannii* ATCC 15151(pOXA66) were very similar to that of *A. baumannii* ATCC 15151(pAT801) except for the conspicuous expression of two proteins of 30 and 32 kDa (Fig. 1, lanes 5 and 6).

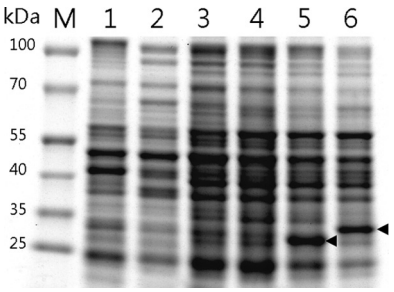

FIG. 1. Expression of $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-66}}$ from *A. baumannii* ATCC 15151. Lane 1, *A. baumannii* 6AB11 (IS*Aba1*-$bla_{\text{OXA-66}}$); lane 2, *A. baumannii* 6AB15 (IS*Aba1*-$bla_{\text{OXA-23}}$ and $bla_{\text{OXA-66}}$); lane 3, *A. baumannii* ATCC 15151; lane 4, *A. baumannii* ATCC 15151(pAT801); lane 5, *A. baumannii* ATCC 15151(pOXA23); lane 6, *A. baumannii* ATCC 15151(pOXA66). The arrows in lanes 5 and 6 indicate the increased expression of 30-kDa and 32-kDa proteins in two transformants. The molecular masses of the size markers (lane M) are given on the left.

Antibiotic susceptibilities of two transformants. We chose the wild-type *A. baumannii* strain ATCC 15151 as the host for investigation of the possible roles of IS*Aba1*-$bla_{\text{OXA-23}}$ and IS*Aba1*-$bla_{\text{OXA-66}}$. The susceptibilities of the isolates to β-lactams are shown in Table 4. Clinical strains 6AB11 and 6AB15 were resistant to all antibiotics tested. *A. baumannii* ATCC 15151 was susceptible to most of the antibiotic agents, and only the MICs of ampicillin, ampicillin-sulbactam, and piperacillin were increased for the transformant *A. baumannii* ATCC 15151(pAT801), harboring the AmpR-selectable marker from pAT801. The imipenem and meropenem MICs for transformants *A. baumannii* ATCC 15151(pOXA23) and ATCC 15151(pOXA66) were significantly increased (64-fold) over that for *A. baumannii* ATCC 15151(pAT801). Furthermore, the piperacillin-tazobactam MICs for those two transformants were increased 4- to 32-fold over that for *A. baumannii* ATCC 15151(pAT801). Only for ATCC 15151(pOXA23) was the cefepime MIC increased (16-fold) over that for the reference strain. ATCC 15151(pOXA23) and ATCC 15151(pOXA66) were susceptible to ciprofloxacin, levofloxacin, amikacin, gentamicin, and tobramycin (data not shown).

DISCUSSION

This study analyzed various genes of *Acinetobacter* clinical isolates that are responsible for resistance to fluoroquinolones, aminoglycosides, cephalosporins, and carbapenems. Although *Acinetobacter* genospecies 3 and *Acinetobacter* genospecies 13TU are genetically closely related to *A. baumannii* and are also associated with nosocomial infections (1), *A. baumannii* is still the predominant genospecies isolated and has a higher ratio of MDR than other *Acinetobacter* spp. Previous studies

TABLE 4. Antibiotic susceptibilities of *A. baumannii* clinical isolates and of *A. baumannii* reference strain ATCC 15151 alone or harboring a recombinant plasmid

β-Lactam(s)	MIC[a] (mg/liter) for:					
	A. baumannii 6AB11 (IS*Aba1-bla*$_{OXA-66}$)	*A. baumannii* 6AB15 (IS*Aba1-bla*$_{OXA-23}$ + *bla*$_{OXA-66}$)	*A. baumannii* ATCC 15151	*A. baumannii* ATCC 15151(pAT801)	*A. baumannii* ATCC 15151(pOXA23) (IS*Aba1-bla*$_{OXA-23}$)	*A. baumannii* ATCC 15151(pOXA66) (IS*Aba1-bla*$_{OXA-66}$)
Ampicillin	≥32	≥32	16	32	≥32	≥32
Ampicillin-sulbactam	≥32	≥32	≤2	16	≥32	16
Piperacillin	≥128	≥128	16	≥128	≥128	≥128
Piperacillin-tazobactam	≥128	≥128	≤4	≤4	≥128	16
Ceftazidime	≥64	≥64	4	4	8	4
Cefepime	≥64	≥64	4	4	≥64	4
Imipenem	≥32	≥32	0.25	0.25	≥16	≥32
Meropenem	≥16	≥16	0.25	0.25	≥16	≥16

[a] The MICs of β-lactams were detected by Vitek 2 except for imipenem, for which the MIC was detected by Etest.

have found differences in antibiotic susceptibility and antibiotic resistance genes among distinct *Acinetobacter* genospecies (16, 19), but they were focused mainly on carbapenem resistance. We found differences in phenotypic and genetic characteristics between *A. baumannii* and *Acinetobacter* genospecies 13TU not only with regard to carbapenem resistance but also with regard to fluoroquinolone, aminoglycoside, and cephalosporin resistance. In our previous studies, ciprofloxacin was the most effective antibiotic against non-*A. baumannii Acinetobacter* isolates(20, 36). In this study, only three non-*A. baumannii Acinetobacter* isolates were ciprofloxacin resistant, and a mutation in Ser83Leu or Ser83Phe was identified, thus further supporting the idea that GyrA mutation was the major mechanism for the resistance of *Acinetobacter* spp. to fluoroquinolones. Although fluoroquinolone-resistant proteins (Qnr) in *A. baumannii* were reported in 2008 (37), this mechanism does not appear to be common in *Acinetobacter* spp. We did not find Qnr-positive isolates within our collections. But this is an interesting topic for further study.

Integrons play an important role in the horizontal spread of antibiotic resistance genes (24). In this study, *aacA4-catB8-aadA1* was the most prevalent (88% [35/40]) integron-borne gene cassette in *A. baumannii*, a finding similar to that of one Taiwanese report (13). *aacA4*, which encoded an aminoglycoside 6′-*N*-acetyltransferase [AAC(6′)-Ib], was shown to confer resistance to amikacin, netilmicin, and tobramycin (34). Although the presence of *aacA4* in *Acinetobacter* spp. was highly correlated with gentamicin and tobramycin resistance, 66% (25/38) of *aacA4*-harboring *Acinetobacter* isolates remained susceptible to amikacin (data not show). Similar findings were obtained in another study (13). In addition, some antibiotic resistance genes were identified on integrons even though their corresponding antibiotics, such as chloramphenicol (*catB8*), rifampin (*arr-3*), trimethoprim (*dhfrXII*), streptomycin, and spectinomycin (*aadA1*, *aadA2*, and *aadA4*), are no longer in use. Such integrons were frequently found in epidemic strains of *A. baumannii* and were associated with a high prevalence of multiple antibiotic resistance, especially aminoglycoside resistance (13, 38).

The basal level of expression of the chromosome-borne *bla*$_{AmpC}$ gene in *A. baumannii* seemed not to reduce susceptibility to expanded-spectrum cephalosporins (2). Our results also indicated that all *bla*$_{AmpC}$-harboring *A. baumannii* isolates were susceptible to cephalosporins, whereas all IS*Aba1-bla*$_{AmpC}$-harboring *A. baumannii* isolates were resistant to ceftazidime, and most of them were not susceptible to cefepime. Although the presence of IS*Aba1-bla*$_{AmpC}$ was correlated with cefepime resistance according to the statistical analysis, other studies showed that the inhibition or overproduction of AmpC had no significant effect on cefepime susceptibility in *A. baumannii* (6, 14). In addition, five IS*Aba3-bla*$_{OXA-58}$-harboring *Acinetobacter* genospecies 13TU isolates resistant to ceftazidime and cefepime without IS*Aba1-bla*$_{AmpC}$ were observed, and it has been suggested that alternative mechanisms may be responsible for resistance to extended-spectrum cephalosporins (3, 6). Among carbapenem resistance genes, we found *bla*$_{IMP-1}$, *bla*$_{VIM-11,}$ and *bla*$_{OXA-58}$ only in *Acinetobacter* genospecies 13TU, while *bla*$_{OXA-51-like}$ was detected only in *A. baumannii*, suggesting that distinct *Acinetobacter* genospecies contained different carbapenemases (17). In other countries, the widespread dissemination of carbapenem-resistant *Acinetobacter* spp. with *bla*$_{OXA-23}$ or *bla*$_{OXA-24}$ has been reported (25, 32). However, we found only 1 *bla*$_{OXA-23}$-harboring and 1 *bla*$_{OXA-24}$-harboring *A. baumannii* isolate in this study, and similar observations have been reported in other Taiwanese studies, suggesting that the presence of IS*Aba1-bla*$_{OXA-51-like}$ was the most common mechanism of carbapenem resistance in *A. baumannii* strains in Taiwan (17, 23). Although IS*Aba1-bla*$_{OXA-51-like}$ was considered a major factor in carbapenem resistance in *A. baumannii*, several *A. baumannii* isolates with IS*Aba1-bla*$_{OXA-51-like}$ remained susceptible to carbapenems in our study. It is likely that differences in transcriptional-level regulation of *bla*$_{OXA-51-like}$ could affect carbapenem susceptibility in different *A. baumannii* strains (12).

In the cloning experiments, both *A. baumannii* ATCC 15151(pOXA23) and *A. baumannii* ATCC 15151(pOXA66) were resistant to carbapenems, indicating that the presence of IS*Aba1-bla*$_{OXA-23}$ or IS*Aba1-bla*$_{OXA-66}$ was sufficient to confer carbapenem resistance even without the assistance of other mechanisms. Figueiredo et al. (8) reported *in vivo* selection of reduced susceptibility to carbapenems in *A. baumannii* associated with the IS*Aba1*-related overexpression of *bla*$_{OXA-66}$. Furthermore, the inactivation of *bla*$_{OXA-66}$ in *A. baumannii* resulted in higher susceptibility to carbapenems, indicating that *bla*$_{OXA-66}$ was involved in reduced susceptibility to carbapenems even when weakly expressed (8). In addition, although

OXA-23 was considered a CHDL, we found that the MIC of cefepime, but not that of ceftazidime, was also significantly increased for *A. baumannii* ATCC 15151(pOXA23) (Table 4). Similar results have been observed previously by introducing a natural bla_{OXA-23}-harboring plasmid, pFER, into *A. baumannii* ATCC 15151 (CIP 70.10) (10), but the authors did not describe these results in detail. Here we introduced IS*Aba1*-bla_{OXA-23} into *A. baumannii* ATCC 15151 (CIP 70.10), and its contribution to cefepime resistance is reported for the first time.

In this study, we showed that the *Acinetobacter* clinical isolates contained various antibiotic resistance genes to combat fluoroquinolones, aminoglycosides, cephalosporins, and carbapenems. Other mechanisms, such as overexpression of an efflux pump and loss of outer membrane protein, were not investigated in this study. Bratu et al. (3) suggested that efflux pumps seemed not to be important contributors to aminoglycoside or fluoroquinolone resistance. Furthermore, nonenzymatic mechanisms were considered not to be major factors contributing to carbapenem resistance (30). Hu et al. (12) determined the transcription level of IS*Aba1*-bla_{OXA-66} in *A. baumannii* by reverse transcription (RT-PCR) and suggested that the expression of bla_{OXA-66} in *E. coli* was correlated with imipenem resistance. However, the direct impact of IS*Aba1*-bla_{OXA-66} in carbapenem-susceptible *A. baumannii* isolates had not been determined. This is the first report directly demonstrating the impact of IS*Aba1*-bla_{OXA-66} in transforming carbapenem-susceptible *A. baumannii* strains to carbapenem-resistant *A. baumannii* strains. In conclusion, *A. baumannii* has been the most successful *Acinetobacter* species in nosocomial infections and contains various antibiotic resistance genes. Continuous surveillance of MDR *Acinetobacter* spp. and elucidation of their antibiotic resistance mechanisms in the hospital are crucial to help develop effective therapy regimens and to prevent the further dissemination of these MDR species.

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