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MECHANISMS OF RESISTANCE

Roles of *ramR* and *tet*(A) Mutations in Conferring Tigecycline Resistance in Carbapenem-Resistant *Klebsiella pneumoniae* Clinical Isolates

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ABSTRACT Tigecycline is regarded as a last-resort treatment for carbapenem-resistant *Klebsiella pneumoniae* (CRKP) infections, but increasing numbers of tigecycline-resistant *K. pneumoniae* isolates have been reported. The tigecycline resistance mechanisms in CRKP are undetermined. This study aimed to elucidate the mechanisms underlying tigecycline resistance in 16 tigecycline- and carbapenem-resistant *K. pneumoniae* (TCRKP) isolates. Mutations in tigecycline resistance determinant genes [*ramR*, *acrR*, *oqxR*, *tet*(A), *tet*(L), *tet*(X), *tet*(M), *rpsJ*] were assessed by PCR amplicon sequencing, and mutations in *ramR* and *tet*(A) exhibited high prevalences individually (81%) and in combination (63%). Eight functional *ramR* mutation profiles reducing tigecycline sensitivity were verified by plasmid complementation of wild-type and mutant *ramR*. Using a site-specific mutant, the most frequent RamR mutation, A19V (60%), had no significant effect on tigecycline susceptibility or the upregulation of *ramA* and *acrA*. Two *tet*(A) variants with double frameshift mutations, type 1 and type 2, were identified; type 2 *tet*(A) is novel. A parent strain transformed with a plasmid carrying type 1 or type 2 *tet*(A) increased the tigecycline MIC by 8-fold or 4-fold, respectively. Synergistic effects were observed in strains harboring no *ramR* gene and a mutated *tet*(A), with an 8-fold increase in the tigecycline MIC compared with that in strains harboring only mutated *tet*(A) being seen. Overall, mutations in the *ramR* and *tet*(A) efflux genes constituted the major tigecycline resistance mechanisms among the studied TCRKP isolates. The identification of strains exhibiting the combination of a *ramR* deficiency and widespread mutated *tet*(A) is concerning due to the possible dissemination of increased tigecycline resistance in *K. pneumoniae*.

KEYWORDS *Klebsiella pneumoniae*, *ramR*, *tet*(A), tigecycline resistance

Tigecycline exhibits expanded-spectrum activity against most clinically significant multidrug-resistant organisms, including carbapenem-resistant *Enterobacteriaceae* (CRE) (1), with the exception of *Proteus* and *Pseudomonas* spp., which are intrinsically tigecycline resistant (2). Carbapenem-resistant *Klebsiella pneumoniae* (CRKP), which has increased in global prevalence over the last decade (3, 4), is the most commonly encountered CRE species (5). Tigecycline is regarded as a last-resort treatment for CRKP infections. However, the emergence of tigecycline-resistant *K. pneumoniae* is a serious problem for the treatment of severe CRKP infections (5, 6).

Tigecycline resistance in *K. pneumoniae* is believed to be primarily mediated by the overexpression of genes encoding the AcrAB-TolC efflux pump, which are controlled by the local repressor *acrR* as well as global transcriptional activators, such as *ramA* (1, 2).

Received 24 February 2017 **Returned for modification** 14 March 2017 **Accepted** 15 May 2017

Accepted manuscript posted online 22 May 2017

Citation Chiu S-K, Huang L-Y, Chen H, Tsai Y-K, Liou C-H, Lin J-C, Siu LK, Chang F-Y, Yeh K-M. 2017. Roles of *ramR* and *tet*(A) mutations in conferring tigecycline resistance in carbapenem-resistant *Klebsiella pneumoniae* clinical isolates. Antimicrob Agents Chemother 61:e00391-17. https://doi.org/10.1128/AAC.00391-17.

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RamR is a local negative regulator of *ramA*; loss-of-function mutations occurring in this protein cause significant increases in *ramA* expression and *acrAB*-dysregulated tigecycline nonsusceptibility (7). The OqxAB efflux pump, which is also associated with tigecycline resistance, is downregulated by the local repressor *oqxR* (8). In addition to efflux-mediated resistance mechanisms, an alteration in the tigecycline target site of ribosomal protein S10, which is encoded by the *rpsJ* gene, has been described (9, 10). There are other known tigecycline resistance mechanisms conferred by Tet proteins, including those associated with the tigecycline-modifying enzyme Tet(X) (11), the mutated efflux pumps of Tet(A) and Tet(L), as well as the ribosomal protective protein Tet(M), that have not yet been identified in *K. pneumoniae* (12, 13).

Cooperative tigecycline resistance mechanisms have emerged and are a cause for concern (14, 15). The combination of mutated *ramR* and *tet*(A) genes was discovered in a tigecycline-resistant *Salmonella enterica* clinical isolate (15), and mutations in the *ramR* and *rpsJ* genes were identified in the whole-genome sequence of a *Klebsiella pneumoniae* carbapenemase (KPC)-producing *K. pneumoniae* isolate (9). To date, only a few functional mutations in tigecycline resistance determinant genes have been identified in studies examining CRKP clinical isolates (16). In addition, there have been no functional studies investigating combined tigecycline resistance mechanisms.

In this study, we collected 16 tigecycline- and carbapenem-resistant *Klebsiella pneumoniae* (TCRKP) clinical isolates to elucidate their tigecycline resistance mechanisms. The two resistance determinants most commonly identified in these isolates, mutated efflux genes *ramR* and *tet*(A), were cloned and transformed into a tigecycline-susceptible wild-type (WT) *K. pneumoniae* clinical isolate to clarify their contributions to tigecycline resistance individually and in combination.

(This study was presented in part as a poster at the 7th International Congress of the Asia-Pacific Society of Infection Control [ASPIC], Taipei, Taiwan, 2015 [17].)

RESULTS

Tigecycline susceptibility testing of TCRKP clinical isolates. The tigecycline MICs and clinical profiles of the 16 TCRKP clinical isolates used in this study are described in Table 1. The strains demonstrating tigecycline MICs of $\geq$4 μg/ml, which were interpreted to be tigecycline resistant following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria, were selected for this study. Of the 16 isolates, 10 (63%) had tigecycline MICs of $\geq$8 μg/ml and 4 (25%) had tigecycline MICs of $\geq$16 μg/ml, including 1 with an MIC of 32 μg/ml.

Identification of tigecycline resistance determinants in TCRKP clinical isolates. Potential tigecycline resistance determinants, specifically, *ramR*, *acrR*, *oqxR*, *tet*(A), *tet*(L), *tet*(X), *tet*(M), and *rpsJ*, were identified in the 16 TCRKP isolates by PCR and sequencing (Table 1). Amplicons for *ramR* were obtained from 14 TCRKP isolates (the exceptions were isolates TR9 and TR12) using published or newly designed primers (see Table S1 in the supplemental material). The genes *acrR*, *oqxR*, and *rpsJ* were detected in all of the isolates; *tet*(A) was found in 13 (81%) isolates (Table 1).

Thirteen isolates (13/16, 81%) showed nucleotide changes in *ramR* or the upstream region in comparison to the sequence of the wild-type *K. pneumoniae* reference strain MGH78578 (GenBank accession number CP000647). The 13 distinct mutation profiles for *ramR* and its upstream sequence are illustrated schematically in Fig. S1 and S2. Six isolates (6/16, 38%) harbored point mutations in the nucleotide sequences upstream of *ramR*, including three mutations in the RamR binding region (G133A in TR8, C126T in TR13, and T125C in TR3) (Fig. S1). Ten isolates (10/16, 63%) harbored frameshift or substitution mutations in RamR (Fig. S2). The most frequent mutation, an A19V substitution, in RamR was observed in six isolates (6/10, 60%) (Table 1). Among isolates with a *ramR* mutation, four isolates harbored substitutions in a DNA-binding domain (A19V and L44R) or a dimerization domain (L111R and E113K). Three isolates with A19V substitutions in RamR also harbored another point mutation (L44R in TR11, L111R in TR1, and E113K in TR2). Truncated RamR proteins were produced by four

TABLE 1 Tigecycline resistance and related characteristics of TCRKP clinical isolates examined in the present study[d]

Isolate	Collection			TGC MIC (μg/ml)	Presence of TGC resistance determinants [mutation(s) occurring in nucleotide or protein sequence][a]								
	Yr	Site	City		*ramR* upstream	*ramR*[b]	*acrR*	*oqxR*	*tet*(A)[c]	*tet*(L)	*tet*(X)	*tet*(M)	*rpsJ*
TR1	2013	Urine	Taipei	32	+	+ (**A19V**, L111R)	+	+	+ (type 1)	−	−	−	+
TR2	2013	Urine	Taipei	16	+	+ (**A19V**, E113K)	+	+	+ (type 1)	−	−	−	+
TR3	2013	Urine	Tainan	16	+ (T125C)	+ (**A19V**)	+	+	+ (type 1)	−	−	−	+
TR4	2012	Bile	Chiayi	16	+	+ (1 nt Δ(415bp))	+	+	+ (type 1)	−	−	−	+
TR5	2012	Urine	Taipei	12	+	+ (stop194K)	+	+	+ (type 1)	−	−	−	+
TR6	2012	Sputum	Taoyuan	12	+ (T55A)	+ (Q122stop)	+ (F204L)	+	−	−	−	−	+
TR7	2012	Urine	Taichung	12	+	+ (**A19V**, Q135stop)	+	+	+ (type 2)	−	−	−	+
TR8	2014	Bile	Kaohsiung	8	+ (G133A)	+	+	+	+ (type 1)	−	−	−	+
TR9	2013	Sputum	Taipei	8	−	−	+	+	+ (type 1)	−	−	−	+
TR10	2012	Urine	Taoyuan	8	+	+ [11 nt Δ(206–216 bp)]	+ (IS*5* insertion)	+ (V130A)	−	−	−	−	+
TR11	2013	Urine	Taipei	6	+	+ (**A19V**, L44R)	+	+	+ (type 1)	−	−	−	+
TR12	2013	Blood	Kaohsiung	6	−	−	+	+	+ (type 1)	−	−	−	+
TR13	2012	Urine	Taipei	6	+ (C126T)	+	+	+	+ (type 1)	−	−	−	+
TR14	2014	Urine	Taipei	4	+ (1 nt Δ(21bp))	+	+	+ (V124D)	+ (type 1)	−	−	−	+
TR15	2012	Sputum	Taipei	4	+	+	+	+	+ (type 1)	−	−	−	+
TR16	2012	Ascites	Chiayi	4	+ (A9T)	+ (**A19V**)	+ (A151V)	+	−	−	−	−	+

[a]Genetic determinants of resistance were detected by performing PCR with gene-specific primers (8, 14, 21, 23). Mutations were determined by comparison with the wild-type reference sequence (*ramR*, *acrR*, and *oqxR* in *Klebsiella pneumoniae* MGH78578 [GenBank accession number CP000647]) or *tet*(A) on plasmid RP1 in *Escherichia coli* [GenBank accession number X00006]).

[b]Bold designations indicate the most frequent point mutation within RamR identified in this study.

[c]Type 1 and type 2 *tet*(A) mutation profiles were designated in this study. The type 1 *tet*(A) sequence was identical to that of the *E. coli* D20-15 RP1 plasmid (GenBank accession number X61367), and type 2 was newly identified in this study.

[d]TGC, tigecycline; Δ, deletion; nt, nucleotide; bp, base pair; +, presence of PCR product and no change in the nucleotide or amino acid sequence; −, absence of PCR product.

isolates, including TR4 (139 amino acids [aa]), TR7 (134 aa), TR6 (121 aa), and TR10 (68 aa) (Fig. S2).

The *acrR*, *oqxR*, and *rpsJ* sequences with mutations were also compared to the MGH78578 reference sequence (GenBank accession number CP000647). Three isolates with *acrR* mutations (F204L in TR6, A151V in TR16, and an IS*5* insertion in TR10) harbored both a *ramR* mutation and *tet*(A), whereas another two isolates with *oqxR* mutations (V130A in TR10 and V124D in TR14) harbored *ramR* mutations only. Among these strains, no mutations in *rpsJ* were detected.

In addition, two Tet(A) variants were identified on the basis of the reference sequences for *Escherichia coli* plasmid RP1 tetracycline resistance determinants (GenBank accession number X00006) and designated the type 1 and type 2 Tet(A) variants. The type 1 Tet(A) variant was found in 12 TCRKP isolates and was identical to that found in the *E. coli* D20-15 RP1 plasmid (GenBank accession number X61367). Only one isolate (TR7) exhibited a novel type 2 Tet(A) variant with a mutation profile of I5R, A93V, G151S, S201A, F202S, V203F, and G268A (Table 1). Both Tet(A) variants harbored double frameshift mutations (S201A, F202S, V203F) (Fig. S3). All 16 TCRKP isolates contained either *ramR* or *tet*(A) mutations, and 13 (81%) TCRKP isolates harbored mutations in both *ramR* and *tet*(A) (Table 1).

Introduction of the *ramR* A19V mutation into the wild-type parent strain. The predominant mutation A19V was introduced into *ramR* in the tigecycline-susceptible parent strain NVT2001, and the isogenic mutant NVT2001::*ramR*-A19V demonstrated a tigecycline MIC of 0.38 μg/ml. The A19V site-specific mutant showed the same MIC values for tigecycline as its parent strain, NVT2001.

Complementation with wild-type *ramR* in TCRKP clinical isolates. To ascertain the effects of *ramR* mutations on tigecycline susceptibility, the 13 TCRKP isolates harboring mutations in *ramR* and/or the region upstream were complemented with wild-type *ramR* and its upstream region. All TCRKP isolates with the exception of three (TR4, TR6, and TR10) showed less than a 4-fold decrease in their tigecycline MICs after introduction of the wild-type *ramR* (Table S2).

Complementation with TCRKP *ramR* in the *ramR* deletion strain. To further elucidate the contribution of *ramR* mutations to tigecycline susceptibility in the 13 TCRKP isolates on an isogenic *K. pneumoniae* strain background, the *ramR* deletion strain NVT2001 Δ*ramR* was constructed. The *ramR* deletion strain had a tigecycline MIC of 3 μg/ml, which was an 8-fold increase compared to the tigecycline MIC for parent strain NVT2001. Eight TCRKP *ramR*-complemented strains showed the same tigecycline MICs as the *ramR* deletion strain, suggesting loss-of-function mutations in their *ramR* genes. Conversely, the other five TCRKP *ramR*-complemented strains had tigecycline MICs that decreased from 3 μg/ml to 0.25 μg/ml, suggesting that their *ramR* mutations had little or no effect on RamR function. The eight functional *ramR* mutation profiles consisted of four nonsense mutations causing premature RamR products, three missense mutations leading to combined point substitutions (A19V and L44R, A19V and L111R, and A19V and E113K), and one nucleotide change in the *ramR* upstream region (C126T) (Table 2).

Relative expression levels of *ramA* and *acrA* in TCRKP isolates and *ramR* mutation strains derived from NVT2001. To speculate on the different genetic changes in *ramR*, the relative *ramA* and *acrA* expression levels of 16 TCRKP isolates were determined by quantitative reverse transcription-PCR (qRT-PCR). Compared to the results for wild-type strain NVT2001, 13 TCRKP isolates overexpressed *ramA*-specific transcripts (9.15- to 59.05-fold) and 3 overexpressed *acrA* (2.18- to 4.1-fold). Among those isolates with the A19V mutation (TR1, TR2, TR3, TR7, TR11, and TR16), not all isolates increased their expression level of *ramA* (0.49- to 59.05-fold) and *acrA* (0.31- to 4.1-fold). In isolate TR15 with wild-type *ramR*, its *ramA* level was high (11.47-fold), while its *acrA* level remained unaffected (Table S3). To further investigate the direct association between the *ramR* mutation profiles and the increased levels of expression of *ramA* and *acrA*, qRT-PCR analysis was performed with a strain into which A19V was

TABLE 2 Tigecycline susceptibility and relative *ramA* and *acrA* expression levels of NVT2001 *ramR* deletion strains harboring wild-type or TCRKP *ramR* plasmids[c]

Strain	*ramR* mutation profile	TGC MIC (μg/ml)	Fold change in expression compared with that for NVT2001 Δ*ramR*/pACYC177[a]	Mean fold change in gene transcription level ± SD[b]	
				ramA	*acrA*
NVT2001	Wild type	0.38	0.13	0.54 ± 0.01	0.83 ± 0.02
NVT2001 Δ*ramR*		3	1	21.2 ± 2.07	3.25 ± 0.45
NVT2001 Δ*ramR*/pACYC177		3	1	23.19 ± 1.35	4.07 ± 0.35
NVT2001 Δ*ramR*/pramR-WT	Wild type	0.25	0.08	1	1
NVT2001 Δ*ramR*/pramR-TR15	Wild type	0.25	0.08	1.22 ± 0.03	0.89 ± 0.36
NVT2001 Δ*ramR*/pramR-TR5	RamR 1-aa extension	0.25	0.08	0.53 ± 0.01	1.61 ± 0.04
NVT2001 Δ*ramR*/pramR-TR14	Upstream 1-bp deletion	0.25	0.08	0.37 ± 0.03	1.21 ± 0.09
NVT2001 Δ*ramR*/pramR-TR8	Upstream substitution G133A	0.25	0.08	0.33 ± 0.01	1.34 ± 0.02
NVT2001 Δ*ramR*/pramR-TR16	RamR substitution A19V + upstream substitution A9T	0.25	0.08	0.92 ± 0.08	1.12 ± 0.02
NVT2001 Δ*ramR*/pramR-TR3	RamR substitution A19V + upstream substitution T125C	0.25	0.08	0.61 ± 0.02	1.25 ± 0.03
NVT2001 Δ*ramR*/pramR-TR10	RamR frameshift mutation	3	1	22.16 ± 0.04	5.36 ± 0.06
NVT2001 Δ*ramR*/pramR-TR4	RamR frameshift mutation	3	1	24.69 ± 1.45	3.99 ± 0.18
NVT2001 Δ*ramR*/pramR-TR6	RamR nonsense mutation + upstream substitution T55A	3	1	24.74 ± 0.11	5.04 ± 0.06
NVT2001 Δ*ramR*/pramR-TR7	RamR nonsense mutation + RamR substitution A19V	3	1	24.25 ± 0.9	5.13 ± 0.11
NVT2001 Δ*ramR*/pramR-TR13	Upstream substitution C126T	3	1	14.32 ± 0.46	8.43 ± 0.04
NVT2001 Δ*ramR*/pramR-TR1	RamR substitutions A19V + L111R	2	0.67	9.62 ± 1	2.21 ± 0.14
NVT2001 Δ*ramR*/pramR-TR2	RamR substitutions A19V + E113K	2	0.67	5.72 ± 0.03	2.62 ± 0.29
NVT2001 Δ*ramR*/pramR-TR11	RamR substitutions A19V + L44R	2	0.67	15.21 ± 1.57	3.7 ± 0.14

[a]Vector-only control.
[b]Measured by qRT-PCR and normalized to the expression levels of NVT2001 Δ*ramR*/pramR-WT (expression level, 1).
[c]TGC, tigecycline; WT, wild type; aa, amino acid; bp, base pair.

incorporated by site-directed mutagenesis and TCRKP *ramR*-complemented *ramR* deletion strains. Increased levels of expression of *ramA* and *acrA* in the A19V isogenic mutant compared to those in its NVT2001 parent strain with wild-type *ramR* were not detected. In addition, in all 8 TCRKP *ramR*-complemented strains that did not show at least a 2-fold reduction in tigecycline MICs, both their *ramA* and *acrA* transcription levels were increased (Table 2).

Introduction of mutated *tet*(A) into the wild-type or *ramR* deletion strain. To evaluate the tigecycline resistance effects of mutated *tet*(A) alone or in combination with a *ramR* mutation, plasmids harboring type 1 or type 2 mutated *tet*(A) were transformed individually into the wild-type or *ramR* deletion strain. The tigecycline MIC of parent strain NVT2001 was 0.38 μg/ml, whereas that of the type 1 *tet*(A)-complemented strain was 3 μg/ml, an 8-fold increase. The type 2 *tet*(A)-complemented strain showed an MIC of 1.5 μg/ml, which was a 4-fold increase. The tigecycline MICs of the type 1 and type 2 *tet*(A)-complemented strains were 24 μg/ml and 12 μg/ml, respectively, which were 63-fold and 32-fold increases, respectively, relative to the tigecycline MIC of the *ramR*-deficient non-*tet*(A)-complemented NVT2001 strain. The *ramR* deficiency increased the tigecycline MICs of the mutants carrying only type 1 or type 2 *tet*(A) by 8-fold, indicating a synergistic mechanism of resistance for tigecycline (Table 3).

DISCUSSION

In this study, the tigecycline resistance determinants detected in 16 TCRKP clinical isolates were efflux-altering mutations, including mutations in *ramR*, *acrR*, *oqxR*, and *tet*(A). Mutations in *ramR* or *tet*(A) were specifically identified in all of the examined strains, suggesting that efflux plays a major role in tigecycline resistance in *K. pneumoniae*. Although various efflux-mediated mechanisms are associated with a low level of resistance to tigecycline (18), it is reasonable to infer that a combination of several efflux mechanisms may exert a synergistic effect on resistance. This study demon-

TABLE 3 Tigecycline susceptibility of NVT2001 harboring the *tet*(A) plasmid alone or in combination with a *ramR* deletion

Strain[a]	TGC[b] MIC (μg/ml)	Fold change in MIC compared with that for NVT2001/pACYC177[c]
NVT2001	0.38	1
NVT2001/pACYC177	0.38	1
NVT2001/ptetA-TR1 (type 1)	3	8
NVT2001/ptetA-TR7 (type 2)	1.5	4
NVT2001 Δ*ramR*	3	8
NVT2001 Δ*ramR*/pACYC177	3	8
NVT2001 Δ*ramR*/ptetA-TR1 (type 1)	24	63
NVT2001 Δ*ramR*/ptetA-TR7 (type 2)	12	32

[a]The type 1 and type 2 *tet*(A) mutation profiles were designated in this study.
[b]TGC, tigecycline.
[c]Vector-only control.

strates, for the first time, that the combination of mutated *tet*(A) and a deficiency in *ramR* has synergistic effects on tigecycline resistance in *K. pneumoniae*.

Mutations in *ramR* are commonly detected in clinical strains of fluoroquinolone-resistant *S. enterica* (19), as well as tigecycline-resistant strains of *Enterobacter cloacae*, *Enterobacter aerogenes* (20), and *K. pneumoniae* (21). *ramR* was not detected in two isolates (TR9 and TR12), suggesting that their *ramR* genes may have been truncated or excised, as has already been observed in *S. enterica* clinical strain 75 (19) as well as complete *ramR* deletion mutants of the *K. pneumoniae* KPBj1 M3 Lev mutant (22). This study identified six mutations located upstream of *ramR*, including one nucleotide deletion at position −21 and five point mutations (A9T, T55A, T125C, C126T, and G133A) that have not been previously identified. Two novel point mutations (L44R and L111R) in the RamR protein were identified, in addition to the previously described A19V (18, 23, 24) and E113K (16) mutations.

The A19V RamR substitution mutation was dominant (4/23, 17%) in a study in China (18); it was also the most frequent mutation (6/10, 60%) found in TCRKP strains harboring mutated RamR. Though at least a 2-fold reduction in the tigecycline MICs was observed in TCRKP strains harboring the A19V change in *ramR* upon complementation with a functional *ramR* (see Table S2 in the supplemental material), not all strains showed a significant upregulation of *ramA* and *acrA* relative to the level of regulation in wild-type strain NVT2001 (Table S3), suggesting that other regulation mechanisms may be involved (8). Our site-directed mutagenesis experiments indicated that the A19V substitution may have no effect on the tigecycline MIC, which is in agreement with the qRT-PCR data showing insignificant *ramA* upregulation in that A19V isogenic mutant relative to its levels of expression in its parent strain with wild-type *ramR* (Table S3), as described in *K. pneumoniae* clinical strain TS238, which harbors this mutation (24).

ramR inactivation appears to be the most prevalent mechanism of tigecycline resistance in *K. pneumoniae* (9), although several natural functional mutations of *ramR*, such as I106F, E113K (16), and S41N plus P46E (24), have been shown to affect *ramA* expression or tigecycline MIC levels in wild-type *ramR*-complemented strains (23–25). Following complementation with a plasmid containing wild-type *ramR*, a high proportion of complemented strains (10/13, 77%) showed less than a 4-fold decrease in their tigecycline MICs (Table S2). In TR15, which has no *ramR* mutation, a 2-fold decrease in the tigecycline MIC value was observed after complementation with wild-type *ramR* (Table S2). The results suggest the presence of other tigecycline resistance determinants (2). Thus, an alternative complementation assay was carried out in which mutated TCRKP *ramR* was introduced into an *ramR* deletion strain. This approach identified eight functional mutations in *ramR* in an isogenic background by determination of tigecycline susceptibility and the relative transcriptional levels of *ramA* and *acrA* (Table 2).

Though all three of these substitutions (C126T in TR13, T125C in TR3, and G133A in

TR8) exist in the RamR binding site, only the C126T mutation upstream of *ramR* in TR13 compromised RamR function, resulting in *ramA* and *acrA* overexpression in the TR13 *ramR*-complemented strain relative to their levels of expression in NVT2001 Δ*ramR*/pramR-WT (Table 2). The result indicated that specific nucleotide mutations in conserved inverted repeat sequences of regulatory elements in the *romA-ramA* locus, such as a 1-bp substitution (26) or a 2-bp deletion (27), may exert various effects on the DNA-binding affinity of RamR (Fig. S1). In addition, four truncated RamR proteins of different lengths (from 68 to 139 aa) in TR4, TR6, TR7, and TR10 may have lost function due to the absence of the α8-α9 region and the interruption of dimerization (Fig. S2) (28). Therefore, the function of the upstream substitution T55A in TR6 with an incomplete RamR lacking the α8-α9 region could not be ascertained in this study. Moreover, further study will be required to determine whether the three novel combined substitutions, A19V and L44R, A19V and L111R, and A19V and E113K, disrupt the RamR protein structure for dimerization or DNA binding.

Additional mutations detected in the Tet(A) major efflux pump of TCRKP type 1 and type 2 *tet*(A)-complemented strains were found to confer tigecycline resistance that may have resulted from double frameshift mutations (S201A, F202S, V203F) within the interdomain loop C3 region of the Tet(A) protein, affecting the substrate specificity of tigecycline (13–15), as previously identified in *Salmonella* species (13). However, in another study, the interdomain loop mutation detected in *E. coli* isogenic mutants had no significant effect on tigecycline susceptibility (29), which may have been attributable to the expression plasmid system or the plasmid copy numbers. Furthermore, type 1 Tet(A) mutations were detected at a high frequency (75%) in the TCRKP isolates and were found in 1,257 *K. pneumoniae* sequences in GenBank (described in Materials and Methods), raising concern over the evolution of tigecycline resistance.

Strains complemented with mutated *tet*(A) and deficient in *ramR* had tigecycline MICs greater than 8 μg/ml and showed an 8-fold increase in tigecycline MICs compared with those of strains deficient in *ramR* only, which is higher than the 2-fold increase reported in *S. enterica* (14). Thus, the coexistence of two mechanisms contributing to low-level resistance may be associated with an increased risk for high-level tigecycline resistance in *K. pneumoniae* clinical isolates.

Conclusion. According to the findings of this study, mutant *ramR* and *tet*(A) tigecycline resistance determinants are prevalent in TCRKP isolates, and their functional gene mutations were identified. Furthermore, an *ramR* deficiency in the presence of a mutated *tet*(A) exerts synergistic effects on tigecycline resistance in *K. pneumoniae*. The combination of a deficiency in *ramR* and widespread mutated *tet*(A) should be of concern due to the potential evolution and dissemination of high-level tigecycline resistance in *K. pneumoniae*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 4. TCRKP clinical isolates were selected from a nationwide surveillance study of carbapenem-nonsusceptible *K. pneumoniae* isolates in Taiwan (30). The TCRKP isolates were collected from patients between January 2012 and December 2014. With the exception of isogenic mutants, the strains were cultured in Luria-Bertani (LB) or brain heart infusion (BHI) medium at 37°C. Transformants harboring antibiotic resistance-conferring plasmids were grown in medium supplemented with 50 μg/ml kanamycin (Sigma-Aldrich, St. Louis, MO) or 100 μg/ml amikacin (Sigma-Aldrich).

Antimicrobial susceptibility testing. Strains were tested for susceptibility to tigecycline by performing an Etest (bioMérieux, Marcy l'Etoile, France) following the manufacturer's instructions, and *Escherichia coli* ATCC 25922 was used as the control strain. Tigecycline resistance was defined as a MIC of at least 4 μg/ml, on the basis of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria ($\leq$1 μg/ml for susceptible, 2 μg/ml for intermediate, $\geq$4 μg/ml for resistant) (31).

Detection and sequence analysis of tigecycline resistance determinant genes. The TCRKP clinical isolates were screened for the tigecycline resistance determinants *ramR*, *acrR*, *oqxR*, *rpsJ*, *tet*(A), *tet*(L), *tet*(M), and *tet*(X) by performing PCR with gene-specific primers (see Table S1 in the supplemental material). The PCR products of the open reading frames (ORFs) and the flanking regions of the target genes were purified with a QIAquick PCR purification kit (Qiagen, GmbH, Germany) and were sent to Genomics Corporation (New Taipei City, Taiwan) for sequencing. Mutations were characterized by comparing the sequences with those of wild-type reference strains [*K. pneumoniae* MGH78578 for *ramR*, *acrR*, and *oqxR* (GenBank accession number CP000647) and *E. coli* plasmid RP1 for *tet*(A) (GenBank

TABLE 4 Strains used in this study

Strain	Relevant characteristic(s)[a]	Source or reference
Klebsiella pneumoniae		
TR1 to TR16	TCRKP clinical isolates collected from a national surveillance study of CRE (2012–2014) in Taiwan	30
NVT2001	Clinical isolate harboring wild-type *ramR*; Amp^r	34
NVT2001::*ramR*-A19V	NVT2001 with RamR-specific point mutation A19V	This study
NVT2001/pA	NVT2001 with pA (Cm^r Tet^r Amk^r), which consists of a pACYC184 backbone and an *armA* amikacin resistance gene cassette	This study
TR1-, TR2-, TR3-, TR4-, TR5-, TR6-, TR7-, TR8-, TR10-, TR11-, TR13-, TR14-, TR15-, TR16/pAramR-WT	The indicated TR isolate with pAramR-WT (*ramR* and its flanking region from a TR isolate cloned into reconstructed pA)	This study
NVT2001 Δ*ramR*	An *ramR* deletion strain derived from NVT2001	This study
NVT2001 Δ*ramR*/pACYC177	NVT2001 Δ*ramR* with pACYC177 (Kan^r Amp^r)	This study
NVT2001 Δ*ramR*/pramR-WT	NVT2001 Δ*ramR* with pramR-WT (wild-type *ramR* and its flanking region cloned into pACYC177 [Kan^r Amp^r])	This study
NVT2001 Δ*ramR*/pramR-TR1, -2, -3, -4, -5, -6, -7, -8, -10, -11, -13, -14, -15, and -16	NVT2001 Δ*ramR* with pramR-TR (*ramR* and its flanking region from a TR isolate cloned into pACYC177 [Kan^r Amp^r])	This study
NVT2001/pACYC177	NVT2001 with pACYC177 (Kan^r Amp^r)	This study
NVT2001/ptetA-TR1 and -7	NVT2001 with ptetA-TR and *tet*(A) and its flanking region from a TR isolate cloned into pACYC177 (Kan^r Amp^r)	This study
NVT2001 Δ*ramR*/ptetA-TR1 and -TR7	NVT2001 Δ*ramR* with ptetA-TR [*tet*(A) and its flanking region from a TR isolate cloned into pACYC177 (Kan^r Amp^r)]	This study
MGH78578	Multiply drug-resistant *K. pneumoniae* strain	ATCC
E. coli S17-1 λ*pir*	*hsdR recA pro* RP4-2 (Tc::Mu Km::Tn*7*) (λ*pir*)	34

[a]WT, wild type; Amp^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Tet^r, tetracycline resistant; Amk^r, amikacin resistant; Kan^r, kanamycin resistant.

accession number X00006)]. The homology and identity of Tet(A) sequences were analyzed using the BLAST online tool at http://www.ncbi.nlm.nih.gov/BLAST/ (accessed 21 January 2017) (32).

Construction of *ramR* deletion mutants. The suicide vector pUT-KB (33), which contains an R6K origin of replication, mobRP4 mobile transfer element, kanamycin resistance cassette, and *sacB* counterselection marker, was used to construct plasmids for *ramR* deletion and site-directed mutagenesis. The nucleotide sequences of *ramR* and its flanking regions in *K. pneumonia*e NVT2001 were identical to those of the wild-type reference strain MGH78578 (GenBank accession number CP000647). NVT2001 was used as the parent strain for the construction of the *ramR* deletion and site-specific mutants. Deletion of *ramR* in the NVT2001 wild-type strain was achieved via in-frame deletion mutagenesis as previously described (34). Briefly, two DNA fragments derived from the regions upstream and downstream of *ramR* were PCR amplified using the ramR-Del-AF/ramR-Del-AR and ramR-Del-BF/ramR-Del-BR primer sets, respectively (Table S1). The two amplicons were spliced to create a *ramR* deletion fragment by overlap extension PCR. The spliced fragment was digested with the ApaI and XbaI restriction enzymes and then cloned into pUT-KB, which was similarly digested, resulting in plasmid pramR-Del. For homologous recombination, pramR-Del was transformed into *E. coli* S17-1 λ*pir* using the heat shock method and was then mobilized into NVT2001 via conjugation. The transconjugants were screened on brilliant green plates containing inositol-nitrate-deoxycholate (BIND) supplemented with kanamycin (50 μg/ml) to select *K. pneumoniae* strains harboring pramR-Del, while the growth of non-*K. pneumoniae* contaminants was effectively suppressed on the BIND plates (33). The selected *K. pneumoniae* strains were incubated for 6 h in 20 ml of BHI medium in the absence of kanamycin, and then the inocula were spread onto LB medium plates containing 10% sucrose. After double-crossover events had occurred, sucrose-resistant and kanamycin-sensitive colonies were selected and screened via PCR and sequencing to identify and verify the mutants with in-frame deletions.

Construction of site-specific mutants. The site-directed mutagenesis of *ramR* was accomplished using a QuikChange II site-directed mutagenesis kit (Agilent, CA, USA). Wild-type NVT2001 *ramR* and its flanking regions were PCR amplified with primers ramR-Sdm-WT-F and ramR-Sdm-WT-R (Table S1), digested with ApaI and XbaI, and then cloned into pUT-KB, which was similarly digested, generating plasmid pramR-Sdm-WT. A single point mutation (A19V) was introduced into *ramR* on pramR-Sdm-WT following the manufacturer's protocol using primers ramR-A19V-F and ramR-A19V-R (Table S1), resulting in plasmid pramR-A19V. Transformation, conjugation, and screening for double-crossover NVT2001::*ramR*-A19V mutants were performed similarly to the procedures for NVT2001 Δ*ramR*, using plasmid pramR-A19V. The presence of the *ramR* A19V mutation in selected mutants was confirmed by DNA sequencing.

***ramR* complementation.** Plasmid complementation studies with wild-type or naturally mutated *ramR* were carried out to validate the functional mutations in *ramR* or the upstream nucleotide sequence. Although multidrug resistance was observed in our TCRKP clinical isolates, these strains remained susceptible to amikacin. The amikacin resistance gene *armA* and its flanking region in an amikacin-resistant extended-spectrum β-lactamase-producing *K. pneumoniae* strain obtained from the Taiwan

Surveillance of Antimicrobial Resistance Project (35) were together amplified with primers armA-F and armA-R (Table S1) and then cloned into the pACYC184 vector using BamHI, generating plasmid pA. Wild-type NVT2001 *ramR* and its flanking regions were PCR amplified with primers ramR-Com-WT-F and ramR-Com-WT-R (Table S1) and cloned into pA using EcoRV, resulting in plasmid pAramR-Com-WT. pAramR-Com-WT was transformed into the TCRKP clinical isolates individually to restore their RamR function. Naturally mutant *ramR* genes and their flanking regions were PCR amplified from the TCRKP isolates with primers ramR-Com-TR-F and ramR-Com-TR-R (Table S1), and each product was individually cloned into pACYC177 using PstI, generating plasmids pramR-TR1, -2, -3, -4, -5, -6, -7, -8, -10, -11, -13, -14, and -16. Each pramR-TR plasmid was transformed into the NVT2001 Δ*ramR* strain to restore the RamR function. Strains NVT2001/pAramR-WT and NVT2001 Δ*ramR*/pramR-TR-WT were used as controls to establish the basal levels of tigecycline susceptibility for comparison with the levels of tigecycline susceptibility of the *ramR*-complemented isogenic mutants.

qRT-PCR. To assess the gene expression levels of *ramA* and *acrA*, quantitative reverse transcription-PCR (qRT-PCR) was performed using a Fast SYBR green PCR master mix (Applied Biosystems) and gene-specific primers (Table S1) in a 7500 Fast real-time PCR system (Applied Biosystems), as previously described (36). Total RNA of the mid-log-phase *K. pneumoniae* inoculum was extracted using an RNeasy kit (Qiagen). cDNA was synthesized from RNase-free DNase I (Qiagen)-treated total RNA using SuperScript III reverse transcriptase (Invitrogen). All qRT-PCR experiments were performed three times, with 23S rRNA being used as an internal control (37).

***tet*(A) plasmid transformation.** Plasmids expressing type 1 and type 2 *tet*(A) variants from the TCRKP isolates were transformed into *tet*(A)-deficient strains NVT2001 and NVT2001 Δ*ramR* to determine the synergism between dysfunctional RamR and mutated *tet*(A). Mutated *tet*(A) and the surrounding regions from isolates TR1 and TR7 were PCR amplified using the primers TetA-Add-F and TetA-Add-R (Table S1) and were cloned individually into pACYC177 with PstI, resulting in plasmids ptetA-TR1 and ptetA-TR7, respectively.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00391-17.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

ACKNOWLEDGMENTS

We are grateful to Jen-Chang Chang, Chin-Lin Huang, and Ya-Wen Hsiao for their excellent technical assistance. We thank Shih-Feng Tsai for providing strain MGH78578. We acknowledge the technical services (real-time PCR system support) provided by the Instrument Center of the National Defense Medical Center.

We have no conflicts of interest to declare.

This study was supported by research grants from the Ministry of Science and Technology (MOST103-2314-B-016-020-MY3, MOST104-2314-B-016-019-MY3, MOST105-2314-B-016-050, MOST105-2320-B-400-016), the Tri-Service General Hospital (TSGH-C106-095, TSGH-C106-097), the Medical Affairs Bureau, Ministry of National Defense, Republic of China (MAB-106-080), and the National Health Research Institutes.

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