Identification of Capsular Types in Carbapenem-Resistant Klebsiella pneumoniae Strains by wzc Sequencing and Implications for Capsule Depolymerase Treatment

Yi-Jiun Pan, Tzu-Lung Lin, Yi-Tsung Lin, Po-An Su, Chun-Tang Chen, Pei-Fang Hsieh, Chun-Ru Hsu, Ching-Ching Chen, Yu-Chia Hsieh, Jin-Town Wang

Department of Microbiology, National Taiwan University College of Medicine, Taipei, Taiwan; Division of Infectious Diseases, Department of Medicine, Taipei Veterans General Hospital, Taipei, Taiwan; Department of Pediatrics, Chang Gung Children’s Hospital, Chang Gung Memorial Hospital, Chang Gung University; College of Medicine, Taoyuan, Taiwan; Department of Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan

Klebsiella pneumoniae is an important human pathogen associated with a variety of diseases, and the prevalence of multidrug-resistant K. pneumoniae (MDRKP) is rapidly increasing. Here we determined the capsular types of 85 carbapenem-resistant K. pneumoniae (CRKP) strains by wzc sequencing and investigated the presence of carbapenemases and integrons among CRKP strains. Ten CRKP strains (12%) were positive for carbapenemase (imipenemase, 6/85 strains; K. pneumoniae carbapenemase, 3/85 strains; Verona integron-encoded metallo-β-lactamase, 1/85 strains). Capsular type K64 accounted for 32 CRKP strains (38%), followed by K62 (13%), K24 (8%), KN2 (7%), and K28 (6%). Sequence types (STs) were determined by multilocus sequencing (MLST), and the results indicated that ST11, which accounted for 47% of these CRKP strains (40/85 strains), was the major ST. We further isolated a K64-specific capsule depolymerase (K64dep), which could enhance serum and neutrophil killing in vitro and increase survival rates for K64 K. pneumoniae-inoculated mice. The toxicity study demonstrated that mice treated with K64dep showed normal biochemical parameters and no significant histopathological changes of liver, kidney, and spleen, indicating that enzyme treatment did not cause toxicity in mice. Therefore, the findings of capsular type clustering among CRKP strains and effective treatment with capsule depolymerase for MDRKP infections are important for capsule-based vaccine development and therapy.

Klebsiella pneumoniae, a Gram-negative bacillus, causes hospital or community-acquired disease (1–3). Most K. pneumoniae strains harbor chromosome-encoded SHV β-lactamase (4). In 1983, the emergence of K. pneumoniae strains producing a mutant of SHV-1 β-lactamase that hydrolyzes extended-spectrum cephalosporins was observed in Germany (5); subsequently, K. pneumoniae strains resistant to third-generation cephalosporins were detected in France, and a new β-lactamase gene closely related to TEM-1 and TEM-2 was identified (6). In 1989, the CTX-M type was reported as a new extended-spectrum β-lactamase (ESBL) family member not belonging to either the TEM type or the SHV type (7), and subsequently it was considered one of the major ESBL types (8). Recent global surveillance data from Europe, North and South America, and Asia revealed that the frequency of ESBL-producing K. pneumoniae was 7.5 to 44% (9). As the prevalence of ESBL-producing isolates increased, carbapenems were used to treat serious infections caused by ESBL-producing K. pneumoniae. However, carbapenem-resistant K. pneumoniae (CRKP) rates have been dramatically increasing worldwide over the past 10 years. In the United States, a significant increase in CRKP prevalence from <1% in 2000 to 8% in 2007 was reported (10). In addition, data from the National Healthcare Safety Network (NHSN) showed that, in 2006 and 2007, CRKP was reported for up to 10.8% of total isolates associated with certain device-related infections (11). In Italy, CRKP increased rapidly from 1 to 2% in 2006 to 2009 to 30% in 2011, as reported by the EARS-Net surveillance system, and from 2% in 2009 to 19% in 2012, as reported by the Micronet surveillance network (12). The incidence of carbapenem resistance among K. pneumoniae cases in intensive care units in Taiwan also increased from 1.2% in 2003 to 11.9% in 2011 (13).

Capsular depolymerases such as bacteriophage-derived endolysidase acting against Escherichia coli K1 and polyglutamic acid degrading enzyme acting against Bacillus anthracis have been reported to prevent bacterial infections in animals by enhancing killing by complement, neutrophils, and macrophages (14–18). Moreover, combination treatment with Aeromonas punctata-derived depolymerase and antibiotic successfully prevented dissemination of K. pneumoniae in mice (19). In our recent study, a K1-specific K. pneumoniae bacteriophage and its depolymerase were shown to be applicable for treatment of K1 K. pneumoniae infections in mice (20). Therefore, understanding capsular type prevalence may be crucial for the development of capsule-based vaccines and phage-
depolymerase treatments that can help with the prevention and treatment of multidrug-resistant *K. pneumoniae* (MDRKP) infections. However, previous serotyping studies yielded limited information because only 25% of hospital infection strains can be typed (21). Recently, a few studies conducted surveys of the capsular types of CRKP by genotyping, and some new capsular types were reported to be predominant in CRKP strains from Italy and from the United States (22–27). Here we used a genotyping method as well as phage-depolymerase typing to clarify the prevalence of capsule types in CRKP from Taiwan and developed a depolymerase-based treatment for the prevalent capsular types in CRKP.

**MATERIALS AND METHODS**

**Bacterial strains.** (i) *Reference strains.* *K. pneumoniae* 77 capsular serotype reference strains were purchased from Statens Serum Institute (Copenhagen, Denmark). Two additional capsular types (KN1 and KN2) that were identified in our previous studies (28, 29) were also included. (ii) *Clinical isolates.* All CRKP strains identified at National Taiwan University Hospital (NTUH), National Cheng Kung University Hospital (NCKUH), and Chang Gung Memorial Hospital (CGMH) between December 2010 and November 2011 in a national cooperative study (project NSC99-3112-B-002-045), as well as all *K. pneumoniae* strains causing bacteremia with carbapenem resistance identified in Taipei Veterans General Hospital (VGH) in 2010 to 2013, were enrolled. These CRKP isolates were selected with regard to their antimicrobial drug resistance by the E-test, with the criteria of MICs of ≥4 μg/ml for imipenem or ≥2 μg/ml for ertapenem (according to CLSI interpretive criteria [30]). A total of 85 CRKP strains were obtained from four hospitals located in northern or southern regions in Taiwan, including 28 strains from NTUH, 17 strains from NCKUH, 2 strains from CGMH, and 38 strains from VGH.

**PCR of carbapenemase genes, β-lactamases, and integrons.** Primers used for detection of genes encoding reported carbapenemases ([31–34], imipenemase (*bla*IPM), Verona integron-encoded metallo-β-lactamase (*bla*VIM), *Klebsiella pneumoniae* carbapenemase (KPC) (*bla*KPC), oxacillinase 48 (*bla*OXA-48), and New Delhi metallo-β-lactamase 1 (*bla*NDM-1), β-lactamases SHV, TEM, CTX-M, and DHA (35), and class 1 integron (36) are listed in Table S1 in the supplemental material. In brief, 3 ml of *K. pneumoniae* cultured overnight was centrifuged at 10,000 × g at room temperature for 10 min. Pellets were resuspended in lysis buffer (B-PERII bacterial protein extraction reagent; Thermo Scientific Pierce, Rockford, IL) and incubated for 30 min. After centrifugation, 30 μl of the supernatant was mixed with 100 μl of a 1-ml solution composed of 3 mg of imipenem monohydrate (Sigma, Saint-Quentin Fallavier, France) (pH 7.8), phenol red solution (prepared as described previously) (37), and 0.1 mmol/liter ZnSO4. After 30 min of incubation, the mixture turned from red to yellow with carbapenemase-producing strains.

**Multilocus sequence typing.** Multilocus sequence typing (MLST) was performed, and data were analyzed using the *K. pneumoniae* MLST website ([http://bigd.db.web.pasteur.fr/klebsiella/klebsiella.html](http://bigd.db.web.pasteur.fr/klebsiella/klebsiella.html)).

**Pulsed-field gel electrophoresis.** The genomic DNA was extracted and pulsed-field gel electrophoresis (PFGE) was performed as described previously (38). Electrophoresis was carried out in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 1.0 mM EDTA [pH 8.0]) using a pulsed-field electrophoresis system, with use of the restriction endonuclease XbaI (New England BioLabs, Ipswich, United Kingdom). Restriction fragments were separated in a 1% agarose gel (Bio-Rad) for 22 h at 200 V and 14°C, with ramp times of 2 to 40 s. Following electrophoresis, the gels were stained with ethidium bromide and visualized under UV light. The banding patterns were interpreted based on the criteria described by Tenover et al. (39).

**Capsular typing by wzy sequencing.** Capsular types of the *K. pneumoniae* strains were determined by wzy genotyping (40). The sequences of the CD1-VR2-CD2 region were compared with those of reference strains (40) by NCBI nucleotide BLAST, and the corresponding capsular types were determined according to the criteria of ≥94% DNA identity for the same types and <80% identity for different types, with reconfirmation by wzy PCR for inconclusive results (e.g., K52 and K79 exhibited 89% wzc DNA sequence identity, and K9 and K45 exhibited 99% wzc DNA sequence identity; the strains were distinguished by wzy PCR [40]). The phylogenetic tree of wzc sequences from reference strains and CRKP strains was generated by the neighbor-joining method using the program MEGA (version 4).

**Sequencing of capsular polysaccharide (CPS) synthesis region.** The *cps* regions of the K64 reference strain and the N386 strain (KN3) were amplified as described previously (29). The products were sequenced by primer walking, providing sequences for the *cps* regions. Genes were annotated by NCBI BLAST.

**wzy PCR genotyping and phage-depolymerase specificity.** Primers specific for the wzy gene of K64 were designed for reconfirmation of the strains with K64 wzy type by wzy PCR (Fig. 1A; also see Table S1 in the supplemental material). Specific primers for strain K59 (N386) were also designed with the intent of confirming the presence of *cps* genes distinct from the 79 documented capsular types (Fig. 1B; also see Table S1 in the supplemental material). In parallel with PCR for strain N386, *cps* PCR genotyping using the same primers was performed with 77 *K. pneumoniae* reference strains (Statens Serum Institute), KN1 (A1517), and KN2 (can0507/1790N). PCR for 23S rDNA was used as a control to confirm that the DNA was amplifiable. K62 and KN3 strains were confirmed with a K62-specific capsule depolymerase and a KN3-specific capsule depolymerase, respectively, KN2 strains were confirmed with KN2 phage and the wzy PCR (28), and K28 strains were confirmed with a K28 phage (our unpublished data).

**K64 phage isolation.** Phages that infected K64 *K. pneumoniae* strains were isolated from untreated water (termed K64-1) (28). *K. pneumoniae* strains were cocultured with untreated water in LB broth overnight. After centrifugation, the supernatant was filtered with a 0.45-μm-pore-size filter and was spotted on LB plates overlaid with K. pneumoniae to detect phage plaques. An agar overlay method was used for isolation of a pure phage preparation and for phage titer determination (41).

**Host ranges of phages.** Host ranges of the isolated phages were determined by spot tests (28). Briefly, LB agar plates were overlaid with top agar...
inoculated with 200 μl of fresh bacterial cultures of K. pneumoniae reference strains. One microliter of phage was spotted on the plate after the top agar with plating bacteria solidified. After 6 to 8 h, plaques were observed when the host was permissive for the phage infection.

**Phage genomic DNA sequencing.** Phage genomic DNA was extracted with a QiaGen Lambda kit (Qiagen, Valencia, CA). After phages were precipitated and lysed, the phage DNA was extracted with phenol-chloroform and then precipitated with ethanol. Genomic sequencing was performed by high-throughput sequencing (Yang-Ming Genome Research Center) using the Illumina/Solexa GAII sequencing platform, with the processing and assembly methods described below. Fifty nanograms of DNA was used to construct a sequencing library by using an Illumina-compatible Nextera DNA sample preparation kit (Illumicore), according to the manufacturer’s instructions. The constructed library was quantified with a bioanalyzer (Agilent) with a high-sensitivity DNA chip. Sequencing was performed by paired-end sequencing, with a 100-bp read length, with a HiSeq 2000 sequencing system (Illumina) at the Genome Research Center of National Yang-Ming University. The sequencing reads were trimmed for quality lower than Q20 and adapters, followed by de novo assembling with CLC Genomics Workbench (CLC bio, Aarhus, Denmark). Coding sequences were further predicted by Vector NTI and annotated by NCBI protein BLAST.

**Capsule depolymerase expression and purification.** The putative capsule depolymerase-encoding gene (K64dep) was cloned into a PET28c plasmid (Novagen, Madison, WI) with a His tag. Primers used for cloning are described in Table S1 in the supplemental material. The resulting plasmid was transformed into an E. coli BL21(DE3) strain. The recombinant His-tagged protein was expressed with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) induction at 25°C for 4 h and then was purified with nickel beads, according to the manufacturer’s instructions (Qiagen) (28).

**Detection of endotoxin.** The Limulus amebocyte lysate (LAL) Kinetic-QCL assay was used to detect endotoxin in purified protein preparations. Reagents were purchased from the Lonza Group, Ltd. (Walkersville, MD), and an ELx808LBS absorbance plate reader (Lonza) was used for detection.

**Alcian blue staining.** Extracellular polysaccharides (containing both capsule and lipopolysaccharide) were extracted with hot phenol, as reported previously (42). The extracted material was incubated for 1 h at 37°C, with or without purified enzyme, and then capsular polysaccharide (CPS) was detected with Alcian blue, as described previously (40).

**Serum resistance assay.** The K. pneumoniae serum resistance assay was performed as described previously (43). A total of 10^8 CFU bacteria was pretreated for 1 h at 37°C with or without K64 capsule depolymerase at a final concentration of 100 μg/ml. Then an inoculum of 2.5 × 10^6 CFU bacteria was mixed with human serum from healthy volunteers at a volume ratio of 1:3. The mixture was incubated for 1 h at 37°C.

**Neutrophil killing assay.** The neutrophil killing assay was performed as described previously (44). Briefly, human neutrophils were freshly isolated from peripheral blood donated by healthy volunteers (43). An inoculum containing 10^7 CFU of bacteria (KCR2A), pretreated for 1 h at 37°C with or without K64 capsule depolymerase at a final concentration of 100 μg/ml, was opsonized with 25% normal human serum on ice for 15 min and was incubated with 10^6 human neutrophils at 37°C for 45 min. Percent survival of depolymerase-treated or untreated bacteria was calculated based on viable counts relative to controls without depolymerase and neutrophils.

**Animal inoculation and capsule depolymerase treatment.** The animal protocol was approved by the Animal Care and Use Committee of NTUH. Five-week-old female BALB/cByl mice were treated with cyclophosphamide at a dose of 100 mg/kg, at 2-day intervals, by intraperitoneal injection. Three days after the second injection of cyclophosphamide, mice were infected with 6 × 10^6 CFU of a K64 K. pneumoniae strain (KCR2A) administered intraperitoneally. One hour, 8 h, or 24 h after the bacterial challenge, the enzyme treatment group was given 150 μg, 37.5 μg, or 18.75 μg purified K64dep protein by intraperitoneal injection (8 mice in each group). The enzyme-treated and untreated groups were monitored for 30 days. The rates of survival were compared and analyzed with the Kaplan-Meier method.

**Acute toxicity.** For the acute toxicity study, 150 μg purified K64dep protein was administered intraperitoneally to normal mice or cyclophosphamide-treated mice (inoculated as described above). After 24 h, one group of mice was sacrificed for blood biochemical analyses and histopathological examinations, whereas the development of abnormal behavior and changes in physical appearance (such as symptoms of hunching and irregular breathing) were observed and the weights of the mice were recorded for 14 days in another group of mice.

**Blood biochemical assays.** Serum was obtained by centrifugation of whole blood at 3,000 × g for 15 min. Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and albumin (ALB) were measured to evaluate the liver function of the mice. Blood urea nitrogen (BUN) and creatinine (Cr) levels were determined for assessment of nephrotoxicity. Cell membrane injury and tissue damage were also evaluated, based on lactate dehydrogenase (LDH) measurements. These biochemical parameters were determined with an Hitachi 7080 biochemical analyzer (Hitachi, Japan).

**Histopathological examinations.** The livers, spleens, and kidneys were harvested, fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histological examinations.

**Nucleotide sequence accession numbers.** The KN2 wzc CD1-VR2-CD2 DNA sequence was submitted to GenBank with accession number AB896250. The sequences of the cps region of the K64 reference strain and the N386 strain (KN5) were submitted to GenBank with accession numbers AB897511 and AB897512, respectively. The genome of the multihost K. pneumoniae bacteriophage K64-I was submitted to GenBank with accession number AB897757. The gene K64dep (2,991 bp in length) was submitted to GenBank with accession number AB897513.

**RESULTS**

**Capsular types of CRKP.** The capsular types of 85 CRKP strains were determined by wzc sequencing (40), and the findings were confirmed by wzy PCR genotyping. Among the 85 strains, 81 strains corresponded to known capsular types (≥96% DNA identity across the CD1-VR2-CD2 variable region of wzc), including 32 (38%) K64, 11 (13%) K62, 7 (8%) K24, 6 (7%) KN2, and 5 (6%) K28 (Table 1; also see Table S2 in the supplemental material). One strain (KCR72) exhibited 88% DNA identity to K52 wzc sequences, but it was subsequently confirmed to be not K52 by wzy genotyping using K52-specific primers (see Table S1 in the supplemental material). In contrast, this strain was positive by K15 wzy genotyping (40) (the K15 reference strain was acapsular and lacked the wzc gene, whereas this strain, KCR72, was capsulated, with evidence of positive string test results [43]). Thus, this strain may possess the same cps as the parental K15 reference strain, and the wzc of this capsulated strain may represent the K15 type. The remaining three CRKP strains, KCR57, KCR58, and KCR59, possessed the same wzc sequences, which were distinct from those of 79 documented capsular types (<80% DNA identity). A phylogenetic tree of wzc sequences from the reference strains and CRKP strains was generated by the neighbor-joining method (see Fig. S1 in the supplemental material). The cps region from one of the three strains (KCR59) was resolved, and the variable region of the cps gene cluster was analyzed (Fig. 1B). Specific primers N386-wzyF and N386-wzyR were designed based on the novel wzy sequence (Fig. 1B; also see Table S1 in the supplemental material); PCR genotyping with this primer pair provided detection only of the three strains with the same wzc sequences, and not any of the
TABLE 1 Prevalence rates of capsular types of carbapenem-resistant K. pneumoniae

<table>
<thead>
<tr>
<th>Capsular type</th>
<th>No. (%) of isolates (n = 85)</th>
<th>ST(s) (no. of isolates)</th>
<th>Carbapenemasea</th>
<th>No. of isolates positive for class 1 integron</th>
</tr>
</thead>
<tbody>
<tr>
<td>K64</td>
<td>32 (38)</td>
<td>ST11 (28), ST20 (1), ST859 (1), ST247 (1), ST1711 (1)</td>
<td>1 (imipenemase)</td>
<td>26</td>
</tr>
<tr>
<td>K62</td>
<td>11 (13)</td>
<td>ST11 (5), ST48 (3), ST378 (2), ST1712 (1)</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>K24</td>
<td>7 (8)</td>
<td>ST45 (6), ST15 (1)</td>
<td>3 (imipenemase)</td>
<td>4</td>
</tr>
<tr>
<td>KN2</td>
<td>6 (7)</td>
<td>ST11 (5), ST716 (1)</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>K28</td>
<td>5 (6)</td>
<td>ST37 (4), ST716 (1)</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>K47</td>
<td>3 (4)</td>
<td>ST11 (1), ST690 (1), ST1714 (1)</td>
<td>3 (KPC)</td>
<td>0</td>
</tr>
<tr>
<td>KN3</td>
<td>3 (4)</td>
<td>ST1 (2), ST1713 (1)</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>K17</td>
<td>2</td>
<td>ST252 (1), ST1715 (1)</td>
<td>1 (imipenemase)</td>
<td>1</td>
</tr>
<tr>
<td>K31</td>
<td>2</td>
<td>ST265 (1), ST690 (1)</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>K2</td>
<td>1</td>
<td>ST65</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>K5</td>
<td>1</td>
<td>ST1718</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>K13</td>
<td>1</td>
<td>ST15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>K14</td>
<td>1</td>
<td>ST1719</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>K15</td>
<td>1</td>
<td>ST11</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>K19</td>
<td>1</td>
<td>ST1720</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>K21</td>
<td>1</td>
<td>ST36</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>K25</td>
<td>1</td>
<td>ST47</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>K27</td>
<td>1</td>
<td>ST36</td>
<td>1 (VIM)</td>
<td>1</td>
</tr>
<tr>
<td>K42</td>
<td>1</td>
<td>ST36</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>K46</td>
<td>1</td>
<td>ST1486</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>K48</td>
<td>1</td>
<td>ST36</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>K54</td>
<td>1</td>
<td>ST1716</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>K24</td>
<td>1</td>
<td>ST1714</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a Genes encoding carbapenemases imipenemase, VIM (Verona integron-encoded metallo-β-lactamase), KPC (Klebsiella pneumoniae carbapenemase), OXA-48 (oxacillinase 48), or NDM-1 (New Delhi metallo-β-lactamase) were detected by PCR.

79 documented capsular types (data not shown). Phage specificity also confirmed this new type, because we have isolated a total of 79 bacteriophages infecting different capsular types of reported reference strains, KN1, and KN2 (our unpublished data), and none of them can infect the three strains (data not shown). Based on these results, we infer that the three strains likely belong to a new capsular type, named KN3.

Prevalence of carbapenemase genes. The prevalence of carbapenemase genes (blaIMP, blaVIM, blaKPC, blaOXA-48, and blaNDM-1) in these CRKP strains was investigated by PCR. Of 85 CRKP strains, 10 isolates (12%) were positive for carbapenemase genes. The positive rates for blaIMP, blaKPC, and blaOXA-48 were 6/85 strains (7%), 3/85 strains (4%), and 1/85 strains (1%), respectively, whereas none of the strains possessed blaOXA-48 or blaNDM-1. The carbapenemase activity of these isolates was assessed by the Carba NP test. All of the 10 strains that possessed genes encoding carbapenemases (blaIMP, blaVIM, or blaKPC) displayed positive reactions (turned from red to yellow), whereas the remaining strains showed negative results (Table 1 and data not shown).

Integron prevalence in CRKP. Integrons are a type of mobile genetic elements thought to be associated with antibiotic resistance, and class 1 integrons were found to be the most common type of integrons in Enterobacteriaceae (45). Therefore, the presence of class 1 integrons in the 85 CRKP strains was investigated. The results showed that class 1 integrons were detected in 73% of CRKP strains (62/85 strains) (Table 1). The integrons carried drug resistance genes such as dihydrofolate reductase genes (dhfr) conferring trimethoprim resistance, gene cassettes associated with resistance to aminoglycosides, including aminoglycoside acetyltransferase genes (aac(6′)-Ib-cr, aac(6′)-Ib-cr, and aad(6)-Ib] and aminoglycoside adenyltransferase genes (aadA1, aadA2, and aadA5), streptothricin acetyltransferase gene (sat), chloramphenicol acetyltransferase genes (cat), rifampin ADP-ribosyl transferase gene (arr), and β-lactamase oxacillinase 1 (blaOXA-1) and oxacillinase 10 (blaOXA-10) genes. Moreover, integrons carrying the carbapenemase gene blaVIM-1 were also found (see Table S3 in the supplemental material).

CRKP sequence types. The sequence types (STs) of all 85 CRKP strains were determined (Table 1; also see Table S3 in the supplemental material). Strains with new MLST profiles were submitted to the Institut Pasteur MLST and whole-genome MLST databases (http://bigdb.web.pasteur.fr) and were assigned to new STs (ST1711 to ST1720). ST11, which accounted for 47% of these CRKP strains (40/85 strains), was the major ST, whereas other sequence types each accounted for less than 10% of CRKP strains. ST11 was also predominant in some capsular types, such as K64 (28/32 strains), K62 (5/11 strains), and KN2 (5/6 strains).

Pulsed-field gel electrophoresis patterns of CRKP strains. We further investigated the genetic diversity of 13 CRKP strains on the basis of their PFGE patterns, to examine the relationships between capsular types, STs, and pulsotypes. We randomly selected 4 ST11 (major ST) strains and 4 non-ST11 strains for K64 capsular types and 3 ST11 strains for three other capsular types (K62, KN2, and K15). In addition, two non-ST11 strains for K62 and KN2 were included for comparison. Strains with differences of fewer than 3 fragments were assigned to the same pulsotypes (similarities of ≧70%). The results revealed that strains with the same ST may belong to different pulsotypes (see Fig. S2, lanes 1 to 4, 9, 11, and 13, in the supplemental material). Conversely, strains designated as the same pulsotype may exhibit different STs (see Fig. S2, lanes 3, 6, 7, and 8, in the supplemental material), either related (e.g., ST11 and ST859 differed only in their tonB alleles) or widely different (e.g., the alleles of the seven MLST genes were all different in ST11 and ST247). In addition, strains of the same ST
that belonged to different capsular types exhibited diverse PFGE patterns (see Fig. S2, lanes 1, 9, 11, and 13, in the supplemental material). PFGE profiles indicated that some strains appeared to be closely related (differences of fewer than 3 fragments) and others showed differences in more than 3 bands (similarities of <70%) or even seven or more bands. The genetic diversity indicates that CRKP strains were not genetically identical clones.

**K64 bacteriophage isolation and capsule depolymerase identification.** A multihost *K. pneumoniae* bacteriophage, K64-1, which can infect K1, K11, K21, K25, K30, K35, K64, and K69 reference strains, was isolated. Phage genome sequences were resolved by high-throughput sequencing. The genome was 346,602 bp in length, and genes were further annotated with NCBI protein BLAST. Multiple putative capsule depolymerase-encoding genes were identified (our unpublished data), and one of these genes was shown to encode a K64-specific depolymerase, as follows. The K64dep gene (2,991 bp in length), located at nucleotides 338917 to 341907 of the K64-1 genome, was cloned and expressed with the His tag. Purified protein was spotted on LB agar plates with the phage hosts. After overnight incubation at 37°C, a semi-clear zone was formed on K64 bacteria (Fig. 2), indicating that the gene (termed K64dep) encodes a K64 capsule depolymerase. K64dep could digest only capsules from strains of the K64 capsular type (including the K64 reference strain and 6 clinical strains) and not the 78 non-K64 reference strains (data not shown).

**Alcian blue staining of enzyme-digested CPS.** The capsule-degrading property of K64dep was further validated by Alcian blue staining of enzyme-treated CPS. Our results revealed that enzyme-free CPS showed high-molecular weight capsular polysaccharide polymers at the top of an SDS-PAGE gel, whereas lower-molecular weight material was observed when CPS was treated with 5 μg of enzyme (Fig. 3).

**Serum sensitivity of enzyme-treated bacteria.** The effects of serum killing after enzyme treatment were assessed in a carbapenem-resistant K64 strain, KCR2A. After incubation with serum for 1 h, almost all of the bacteria were killed by 75% serum when the bacteria were pretreated with K64dep (Fig. 4), whereas the bacterial load was moderately decreased to about 40% of the initial inoculum in control samples. Compared to control samples, which can proliferate with 10% and 25% serum, the enzyme-treated bacteria were sensitive to serum killing (Fig. 4). Thus, the capsule depolymerase can enhance bacterial susceptibility to serum killing.

**Neutrophil killing of enzyme-treated bacteria.** Neutrophil-mediated killing of the K64 strain KCR2A, with or without enzyme treatment, was examined. Compared to untreated controls, the numbers of bacteria pretreated with K64dep enzyme were significantly decreased after incubation with human neutrophils.

**FIG 2** Spot test of the K64-1 phage and K64 capsule depolymerase. The K64 reference strain was grown on a LB plate. Phage (10⁶ PFU) or various amounts of capsule depolymerase were spotted on the plate, and plaques or a semi-clear zone, respectively, was observed. The amounts of enzyme used are shown in parentheses.

**FIG 3** Alcian blue staining of CPS treated with K64 capsule depolymerase. Polysaccharide extracts from the K64 reference strain and a carbapenem-resistant K64 strain, KCR2A, were treated with 5 μg K64dep and stained with Alcian blue (see Materials and Methods). Lane M, protein markers.

**FIG 4** Survival of enzyme-treated bacteria with serum killing. Bacterial susceptibility to killing by human serum for a carbapenem-resistant K64 strain, KCR2A, with or without enzyme (enz) pretreatment, is presented. Survival ratio, percent survival of bacteria after a 1-h incubation with serum (viable counts relative to the initial inoculum). Three independent experiments were performed, and the survival rates of the two groups (control and enzyme pretreated) were compared by Student’s *t* test (75% serum, *P* = 0.0002; 25% serum, *P* = 0.0018; 10% serum, *P* < 0.0001). ***, *P* < 0.01; ****, *P* < 0.001.
suggesting that enzyme treatment makes bacteria more susceptible to neutrophil killing ($P = 0.0004, t$ test).

Capsule depolymerase treatment in *K. pneumoniae*-infected mice. Since K64 strains were less virulent than strains causing community-acquired pyogenic liver abscess (PLA) (for example, the 50% lethal dose [LD$_{50}$] of NTUH-K2044 for mice was $\sim 1 \times 10^3$ CFU [20]) and exhibited very high lethal doses (LD$_{50}$ of 3 $\times 10^7$ CFU), we assessed the therapeutic effect of capsule depolymerase in mice treated with cyclophosphamide, which reduces the numbers of white blood cells in mice [20]. The results showed that 6 of the 8 mice infected with $6 \times 10^6$ CFU of a CRKP K64 strain (KCR2A) died within 2 days without enzyme treatment. In contrast, infected mice treated with various amounts of enzyme at 1 h postinfection all survived and appeared to be healthy after 30 days. Statistical analysis of survival rates revealed that enzyme treatment was therapeutic for MDRKP infections ($P = 0.0029, \text{log-rank test}$). However, the enzyme became therapeutically ineffective when it was administered 8 h postinfection ($P = 0.09, \text{log-rank test}$) (Fig. 6).

**Acute toxicity study.** The amounts of endotoxin in purified protein preparations were determined with K64dep levels of 9 endotoxin units (EU)/mg. In order to evaluate the acute toxicity caused by enzyme injection, blood biochemical analyses and histopathological examinations were conducted with the following four groups: normal mice, normal mice treated with enzyme (150 $\mu$g), cyclophosphamide-treated mice, and cyclophosphamide-treated mice treated with enzyme. Analysis of variance (ANOVA) showed that there were no differences in serum levels of AST, ALT, ALB, BUN, Cr, and LDH between the four groups 24 h after enzyme treatment (see Fig. S3 in the supplemental material). Livers, spleens, and kidneys did not show significant histopathological changes in the enzyme-treated mice (see Fig. S4 in the supplemental material). In addition, during the 14-day observation period, no obvious differences in weight gain were found between the control and enzyme-treated groups (data not shown), and no development of abnormal behavior, changes in physical appearance, or any other adverse reactions were observed. Therefore, enzyme treatment did not cause toxicity in mice.

**DISCUSSION**

*K. pneumoniae* causes hospital-acquired infections (1) as well as community-acquired pneumonia (2) and pyogenic liver abscess (PLA) (3). The capsule has been regarded as a major virulence factor of *K. pneumoniae*, and capsular types are related to the clinical manifestation of infections (43, 46). The prevalence of capsular types in clinical settings of community-acquired infections, such as PLA (29, 47, 48) and pneumonia (2), was relatively clear, while types in hospital-acquired infections remained unclear, due to the limitations of traditional serotyping ($\sim 25\%$ were typeable) (21, 49, 50). K1 and K2 were the predominant capsular types of PLA strains (60 to 85%) (47) and pneumonia strains (53%) (2). Nevertheless, capsular types of CRKP were rarely documented, because CRKP studies focused in hospital-acquired infections. A recent report that directly resolved the CPS structures of two CRKP outbreak isolates and characterized them as a new type also noted that difficulties in serotyping could be the reason for the unavailability of serotype data for CRKP (22). Subsequently, the new capsular type (cpsBO-4-like) was reported to be predominant in *K. pneumoniae* carbapenemase (KPC)-producing *K. pneumoniae* strains (38/46 strains [82.6%]) and the other new capsular type (cps207-2-like) accounted for 17.4% of the 46 CRKP
strains from a collection of *K. pneumoniae* clinical isolates in Italy (23). The two new types were also considered to be major types in epidemic KPC-producing *K. pneumoniae* ST258 strains in the United States (25–27). Another study documented that most of the CRKP strains from New York were assigned to a ST258 clone by multilocus sequence typing (MLST) (32/40 strains [80%]), but capsular types were not well defined and several novel C-patterns of *cps* synthesis regions were identified (24, 51).

Here we determined the capsular types of CRKP in Taiwan using *wzc* sequencing and confirmed the findings with *wzy* PCR or phage-depolymerase specificity assays. K64, which accounted for 32/85 CRKP strains (38%), was the most prevalent type. K62 was the second most common type (11/85 strains [13%]), followed by K24 (7/85 strains [8%]), KN2 (6/85 strains [7%]), and K28 (5/85 strains [6%]). These 5 capsular types accounted for 72% of CRKP strains (61/85 strains). Intriguingly, although K1 and K2 seem to be the most common capsular types causing PLA or pneumonia, there was no K1 strain and only one K2 strain among the CRKP isolates. The results indicated that MDRKP (mostly from nosocomial infections) showed different patterns of capsular types than community-acquired isolates. Similar observations were noted in a report (21) that indicated that isolation of the K1 serotype in community-acquired infections was higher than in nosocomial infections (30% versus 14%). Therefore, our important finding of the clustering of capsular types in CRKP could be an important basis for development of capsule-based vaccines and phage-depolymerase treatments. In addition, three of the CRKP strains could belong to a new capsular type, according to their novel *wzc* sequences (40). We further resolved the *cps* region of the type and found that its *cps* sequences were distinct from those of 79 existing capsular types and the reported predominant two new types of KPC-producing strains from Italy and the United States (the *wzc* alleles of the previously reported two types did not match any reference or currently resolved *wzc* sequences, i.e., <80% DNA identity) (23, 25–27). Moreover, the results from the current study indicate that *wzc* sequencing is a useful method for capsular genotyping.

To examine the genetic diversity and relationships among the CRKP strains, STs, pulsortypes, and the sequences of various genetic loci were determined (see Table S3 in the supplemental material). Previous studies reported that ST258 is a major clone throughout the world (23, 24, 52), and a single-locus variant of ST258, ST11, was considered to be predominant in ESBL-producing strains in Asia (53, 54) and was prevalent in CRKP strains in Taiwan (55). In the current study, ST11 was the major ST of these CRKP strains (40/85 strains [47%]), whereas other STs each accounted for less than 10% of CRKP strains. Moreover, ST11 was also predominant in some capsular types, such as K64 (28/32 strains), K62 (5/11 strains), and KN2 (5/6 strains).

PFGE analysis of 13 CRKP strains revealed that strains with the same ST may belong to different pulsortypes. Conversely, strains designated as the same pulsortype may exhibit different STs. In addition, strains with the same ST but different capsular types exhibited diverse PFGE patterns. We also found that, even when strains were assigned to the same ST and capsular type, there was obvious genomic heterogeneity (e.g., PFGE patterns, integrin-carrying genes, β-lactamases, and MICs) among the strains (see Table S3 in the supplemental material). The genetic diversity indicated that the collected strains were not from an outbreak of a single clone, and we suggest that carbapenem-resistant *K. pneum-
endotoxin in the current study. The capsule-degrading property of K64dep was validated by Alcian blue staining of enzyme-treated CPS at low molecular weight. The difference between K64ref and KCR2A after enzyme treatment could be due to different CPS amounts from bacteria cultured overnight. The serum killing and neutrophil killing experiments indicated that capsule depolymerase could efficiently reduce the bacterial resistance against serum and neutrophils. Because K64 strains were less virulent than strains causing community-acquired pyogenic liver abscess and had very high lethal doses, a high dose of K. pneumaniae (6 x 10^6 CFU) was given to mice. The results indicated that the therapeutic effect of capsule depolymerase in cyclophosphamide-treated mice was very significant, but capsule depolymerase was ineffective if administered 8 h postinfection. A possible reason could be too many bacteria after rapid proliferation in cyclophosphamide-treated mice. K64dep-producing phages or combination treatment might overcome this problem.

The toxicity study demonstrated that mice treated with 150 μg of K64dep, which contained 1.36 EU of endotoxin (9 EU/mg K64dep), showed normal biochemical parameters (AST, ALT, ALB, BUN, Cr, and LDH levels) and no significant histopathological changes in liver, kidney, and spleen. Therefore, enzyme treatment did not cause toxicity in mice. However, for application in human therapy, endotoxin can be further removed and reduced to lower levels (~0.2 EU/mg) using more effective methods, as described previously (59). The efficacy and safety results indicated that the depolymerase could be an alternative therapy for MDRKP infections.

In conclusion, we reveal a clustering of CRKP capsular types and develop a depolymerase-based nonantibiotic treatment. Capsular type prevalence data could also facilitate the development of capsule-based vaccines for MDRKP.

ACKNOWLEDGMENTS

We thank the team of curators of the Institut Pasteur MLST system (Paris, France) for importing novel profiles and isolates at http://bigdb.web.pasteur.fr.

This study was supported by grants from the National Science Council, National Taiwan University, National Taiwan University Hospital, and the Liver Disease Prevention and Treatment Research Foundation in Taiwan.

REFERENCES


