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# Sequencing and Comparative Genomic Analysis of pK29, a 269-Kilobase Conjugative Plasmid Encoding CMY-8 and CTX-M-3 β-Lactamases in *Klebsiella pneumoniae*▽

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**A 269-kilobase conjugative plasmid, pK29, from a *Klebsiella pneumoniae* strain was sequenced. The plasmid harbors multiple antimicrobial resistance genes, including those encoding CMY-8 AmpC-type and CTX-M-3 extended-spectrum β-lactamases in the common backbone of IncHI2 plasmids. Mechanisms for dissemination of the resistance genes are highlighted in comparative genomic analyses.**

Plasmid-mediated β-lactamases play a key role in the increasing multidrug resistance in the *Enterobacteriaceae* worldwide, among which CTX-M-type extended-spectrum β-lactamases (ESBLs) and AmpC-type β-lactamases are two major contributors in recent years (3, 14, 17, 19). In general, ESBLs confer resistance to oxyimino-cephalosporins but not cephamycins and are inhibited by β-lactamase inhibitors, while AmpC-type β-lactamases provide resistance to cephamycins and oxyimino-cephalosporins and are refractory to β-lactamase inhibitors. Although reports of multiple β-lactamases in a single pathogen are increasing for the *Enterobacteriaceae*, especially *Klebsiella pneumoniae* (1, 9, 15, 22, 26), the complete sequence information for a plasmid that encodes both an ESBL and an AmpC-type β-lactamase has not been reported.

In Taiwan, CTX-M- and SHV-type ESBLs and CMY- and DHA-type AmpC-type enzymes are the most common β-lactamases that can confer resistance to extended-spectrum cephalosporins in clinical *K. pneumoniae* isolates (24, 26). Here we report the sequencing, annotation, and comparative genomic analysis of an IncHI2 plasmid isolated from a nosocomial *K. pneumoniae* strain. The plasmid carries both CTX-M- and CMY-type β-lactamase genes.

Three *K. pneumoniae* isolates from three patients were collected from the National Cheng Kung University Hospital in Taiwan during a 1-month period in 2001. These three isolates shared identical antimicrobial susceptibility and plasmid profiles (data not shown). Conjugal transfer was performed by using one of the *K. pneumoniae* isolates, NK29, as the donor and *Escherichia coli* J53 Azi$^r$ as the recipient, following a previously described protocol (11). Transconjugants were obtained at an efficiency of $10^{-4}$ to $10^{-5}$ transconjugants/donor at 25°C. DNA sequencing of the plasmid was determined as part of the process of sequencing the entire genome of *K. pneumoniae* NK29, using a shotgun approach. Sequence assembly, annotation, and analysis followed previously described protocols (4).

The plasmid pK29 was determined to be a 269,674-bp circular plasmid with a G+C content of 46%. A total of 310 open reading frames (ORFs) were identified. Since pK29 carries the backbone of the IncHI2 plasmids, we compared its nucleotide sequence with those of pR478 and pAPEC-O1-R, the IncHI2 plasmids with complete nucleotide sequences available to date. Plasmid pR478 (275 kb) was from *Serratia marcescens* clinical isolates, and plasmid pAPEC-O1-R (241 kb) was from avian pathogenic *E. coli* strains (6, 12).

The common regions of the three plasmids contain essential genes for plasmid replication, maintenance, and transmission. These include the *repHIA* and *repHI2* regions for replication initiation, the *trh* and *tra* genes for conjugation, and the *par* region for plasmid segregation (Fig. 1).

Two antimicrobial resistance gene-containing regions (Fig. 1, regions 1 and 2) are unique to pK29. Region 1 (bp 94,051 to 115,352) encodes HipBA multidrug tolerance protein homologs (13) and carries a type I integron containing the antimicrobial resistance gene cassettes *aadA2*, *aac* (putative), and *catB2* (Fig. 2). In the integron region, the inverted repeat IRt was identified at the right end of IS*6100* but the IRi was not found. The integron is flanked by two complete IS*26* elements. In addition, a partial duplicate of the 3′-conserved sequence of the integron, including the *qacEdelta1* and *sul-1* genes, was identified on the left of the integron, followed by an AmpC β-lactamase gene, $bla_{CMY-8}$. No *intI1* or gene cassettes were found on the left of the duplicate 3′-conserved sequence.

The AmpC β-lactamase $bla_{CMY-8}$ was first identified in a 25-kb plasmid of a *K. pneumoniae* epidemic strain from southern Taiwan (25). The originally determined 1,973-bp

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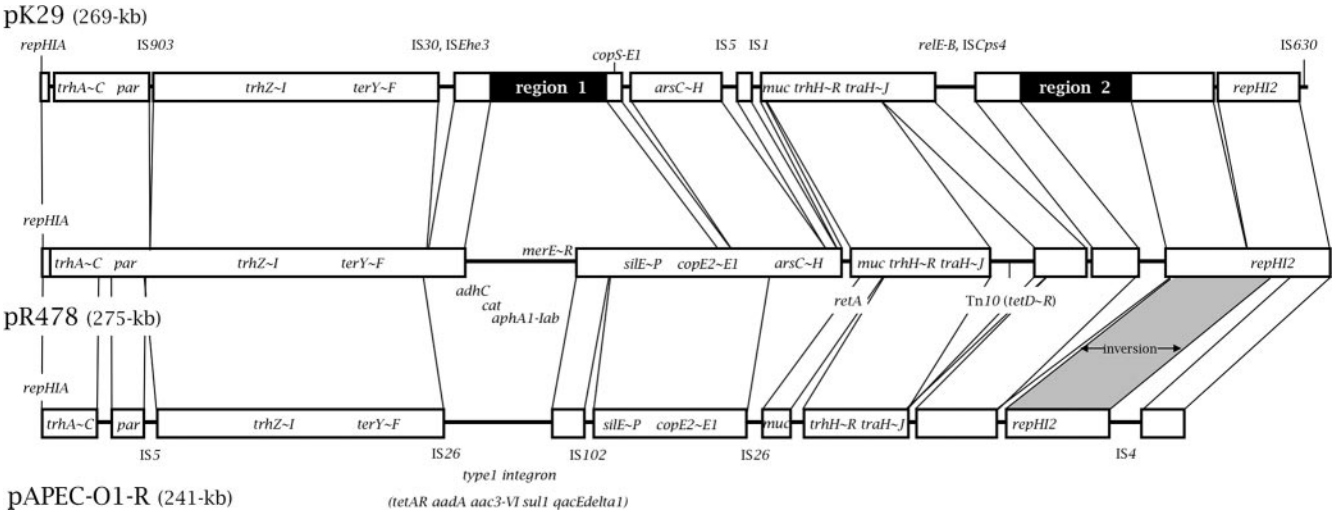


FIG. 1. Map of pK29 and comparison of pK29 with the other two IncHI2 plasmids, pR478 and pAPEC-O1-R. The lines between the three plasmids indicate the homologous sequence blocks which are >99% identical (white squares). Inversion between the homologous regions is shaded. The important features are indicated, as follows: *repHIA* and *repHI2*, replication origins; *trh* and *tra*, conjugal transfer genes; *parAB*, plasmid partitioning genes; *muc*, *mucAB* mutagenesis genes. The *ter*, *ars*, *cop*, *sil*, and *mer* genes encode products conferring tellurite, arsenic, copper, silver, and mercury resistance, respectively. Other genes and their products are as follows: *adhC*, alcohol dehydrogenase; *cat*, putative chloramphenicol acetyltransferase; *aphA1-Iab*, aminoglycoside phosphotransferase; *tet*, tetracycline resistance proteins; *aadA*, putative streptomycin adenyltransferase; *aac3-VI*, gentamicin resistance protein; *sul-1*, dihydropteroate synthetase; and *qacEdelta1*, quaternary ammonium transporter protein. The two regions unique to pK29 (regions 1 and 2) are shown in black.

spanning sequence of $bla_{CMY-8}$ (GenBank accession no. AF167990), which contains part of the upstream *orf513* and the downstream hypothetical ORF, can be aligned perfectly to that of pK29. The association of $bla_{CMY-8}$ with *orf513* (recently renamed insertion sequence common regions [IS*CR1*]) (23) indicates that the $bla_{CMY-8}$ gene is mobilized with these type I integron-associated elements. In pK29, an additional IS*26* element was identified at the left end of *orf513* (Fig. 2). Thus, the $bla_{CMY-8}$ region and the nearby type I integron region represent two small composite transposon-like elements, and the entire 14-kb region bounded by the two external IS*26* elements could also be regarded as a large composite transposon (Fig. 2). We did not, however, find direct repeats or other supportive evidence for the transpositional acquisition of any of these putative composite transposons. The insertion sequences likely contributed to the formation of the region as a result of multiple transposition and recombination events.

Region 2 (bp 208,636 to 230,024) contains a *lacIZY* gene cluster followed by several IS-related conserved hypothetical genes and a 3.8-kb putative composite transposon. This contains a $bla_{CTX-M-3}$ ESBL gene flanked by two convergently

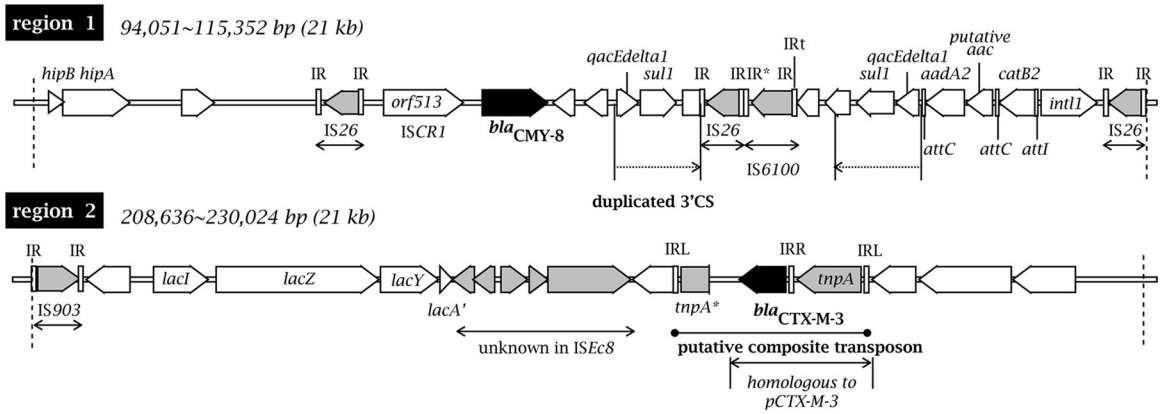


FIG. 2. Schematic diagram of regions 1 and 2, which are unique to pK29. The boundaries between the regions and the known sequence from pR478 are indicated by dashed lines. The ORFs are shown as arrows, with the arrowheads indicating the direction of transcription. The β-lactamase genes are shown in black. The ORFs related to IS are shown in gray. Unmarked white arrows are hypothetical genes. IR, inverted repeats; IR*, incomplete inverted repeats. The genes and their products are as follows: *sul-1*, dihydropteroate synthetase; *qacEdelta1*, quaternary ammonium transporter protein; *aadA2*, streptomycin adenyltransferase; putative *aac*, putative aminoglycoside 6′-*N*-acetyltransferase (80% identical to AAC6-II of *Acinetobacter baumannii* [GenBank accession no. ABL95928]); *catB2*, chloramphenicol acetyltransferase; and *intI1*, integrase. In region 2, the IS*Ecp1* element to the left of $bla_{CTX-M-3}$ is truncated (*tnpA**). The region homologous to plasmid pCTX-M-3 is indicated.

TABLE 1. MICs of different antimicrobials and ESBL test results for the clinical strain *K. pneumoniae* NK29, its plasmid pK29 transconjugant *E. coli* J53/pK29, and the reference strain *E. coli* J53

| Antimicrobial agent | MIC (μg/ml)[a] | | | Zone of inhibition (mm) by disk diffusion test | | |
|---|---|---|---|---|---|---|
| | *K. pneumoniae* NK29 | *E. coli* J53/pK29 transconjugant | *E. coli* J53 | *K. pneumoniae* NK29 | *E. coli* J53/pK29 transconjugant | *E. coli* J53 |
| Aminoglycosides | | | | | | |
| Gentamicin | 3 | 1.5 | 0.25 | | | |
| Kanamycin | 32 | 32 | 8 | | | |
| β-Lactams | | | | | | |
| Amoxicillin-clavulanic acid | 48 | 48 | 8 | | | |
| Aztreonam | 4 | 2 | 0.064 | | | |
| Cefepime | 2 | 1 | 0.094 | | | |
| Cefotaxime | 32 | 24 | 0.064 | | | |
| Cefoxitin | >256 | >256 | 4 | | | |
| Ceftazidime | 16 | 1.5 | 0.19 | | | |
| Ceftriaxone | 24 | 16 | 0.19 | | | |
| Imipenem | 0.25 | 0.25 | 0.25 | | | |
| Other drugs | | | | | | |
| Chloramphenicol | 96 | 96 | 4 | | | |
| Ciprofloxacin | 0.047 | 0.012 | 0.012 | | | |
| Sulfamethoxazole | >512 | >512 | ≤64 | | | |
| Tetracycline | 8 | 2 | 2 | | | |
| Trimethoprim-sulfamethoxazole | 0.50 | 0.064 | 0.047 | | | |
| Drugs for ESBL testing[b] | | | | | | |
| CAZ/CAZ + CLA | 16/>4.0 | 1.5/1.0 | <0.5/0.25 | | | |
| CTX/CTX + CLA | >16/>1.0 | >16/>1.0 | <0.25/0.064 | | | |
| CAZ vs CAZ + CLA | | | | 17 vs 17 | 27 vs 27 | 33 vs 33 |
| CTX vs CTX + CLA | | | | 13 vs 15 | 16 vs 17 | 31 vs 31 |

[a] Based on Etest results, except for kanamycin and sulfamethoxazole MICs, which are from broth microdilution tests. For sulfamethoxazole, the lowest concentration tested was 64 μg/ml, and thus the actual MIC for J53 could be lower.

[b] CAZ, ceftazidime; CTX, cefotaxime; CLA, clavulanic acid. The ceftazidime results were all negative, and the cefotaxime results were indeterminate for NK29 and the J53/pK29 transconjugant based on MIC results. The isolates all tested negative for ESBLs by the CLSI disk diffusion confirmatory test (5).

positioned IS*Ecp1* elements at both ends, with that on the left being truncated (Fig. 2).

The CTX-M-3 ESBL was first found among clinical *Enterobacteriaceae* isolates from Warsaw (7) and, soon after, from Taiwan (27). The finding of identical CTX-M enzymes in widely separated parts of the world has been suggested to be the result of independent evolution (17). However, the $bla_{\text{CTX-M-3}}$ genes of both pK29 and pCTX-M-3, the plasmid from Poland (GenBank accession no. AF550415), were found to reside in a 3-kb homologous region with >99% identity, including a 1,656-bp IS*Ecp1* element upstream of the β-lactamase gene. Recent studies on the frequent association of $bla_{\text{CTX-M}}$ and IS*Ecp1*-like insertion sequences have demonstrated that the IS may be responsible for the mobilization and expression of the β-lactamase gene (20, 21). Thus, the presence of $bla_{\text{CTX-M-3}}$ in different geographical regions is likely to be the consequence of horizontal transmission assisted by the nearby IS*Ecp1* element.

The plasmid pK29 carries both *hipBA* and *relEB* homologs. Known as type II toxin-antitoxin modules, the products of these genes are important in the segregation maintenance of plasmids (8, 10). Recently, some of them have been suggested to play a role in the formation of persister cells that exhibit multidrug tolerance (13). pK29 also carries a *mucAB* operon for mutagenesis (16). By facilitating the formation of persister cells and enhancing the mutation rate, these genes may provide additional advantages to bacterial survival in the presence of lethal factors such as antibiotics.

The transconjugant *E. coli* J53/pK29 revealed resistance or decreased susceptibilities to cefotaxime, cefoxitin, ceftriaxone, amoxicillin-clavulanic acid, aztreonam, ceftazidime, cefepime, chloramphenicol, and sulfamethoxazole (Table 1). The resistance phenotype is consistent with the resistance genes identified on the plasmid. Both NK29 and the J53/pK29 transconjugant tested negative for ESBL by the CLSI ESBL confirmatory disk diffusion test (5), likely due to the presence of the AmpC β-lactamases. Thus, the coexistence of ESBL and AmpC β-lactamases not only limits treatment options but also complicates routine phenotypic detection of ESBLs, a problem of increasing concern for clinical microbiology laboratories (2, 17, 18).

This is the first report of a completely sequenced plasmid that carries both CTX-M-type ESBL and CMY-type AmpC β-lactamase genes. The finding of the coexistence of these genes in such a transmissible plasmid that can propagate in different hosts provides further insight into the mechanisms of transmission of these β-lactamases in Taiwan, where plasmid-mediated ESBLs and AmpC β-lactamases are prevalent.

**Nucleotide sequence accession number.** The annotated sequence of pK29 has been submitted to the GenBank nucleotide sequence database under accession number EF382672.

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