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Cloning of a Cation Efflux Pump Gene Associated with Chlorhexidine Resistance in *Klebsiella pneumoniae*

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Expression libraries of a chlorhexidine-resistant *Klebsiella pneumoniae* strain were constructed and transformed into *Escherichia coli* XLOLR. Twenty chlorhexidine-resistant transformants were obtained after selection. All clones contained a novel 903-nucleotide locus. Its sequences were compatible with a cation efflux pump, and the locus was thus designated as *cepA*. Retransformation using *cepA*-containing plasmids conferred chlorhexidine resistance to both XLOLR and a chlorhexidine-sensitive *K. pneumoniae* strain. Therefore, CepA is associated with chlorhexidine resistance and may act as a cation efflux pump.

Chlorhexidine is an extensively used handwashing antiseptic (9, 12, 18). Some hospital-acquired gram-negative bacteria, including *Klebsiella*, *Serratia*, *Pseudomonas*, etc., are resistant to chlorhexidine (4, 6, 11, 21, 23). Among these bacteria, *Klebsiella pneumoniae* frequently causes pneumonia, urinary tract infection, wound infection, and bacteremia in hospitalized patients (5, 15). The mechanisms responsible for chlorhexidine resistance in gram-negative bacteria remain unclear. Therefore, we tried to isolate the gene(s) responsible for chlorhexidine resistance in *K. pneumoniae* by using λ-Zap II expression libraries.

K. pneumoniae isolates were collected at National Taiwan University Hospital. Chlorhexidine MICs were determined by agar dilution techniques (13) by using chlorhexidine digluconate (Sigma, St. Louis, Mo.). Fifty randomly selected clinical *K. pneumoniae* isolates were tested for chlorhexidine MICs. Chlorhexidine had a MIC of ≥32 μg/ml for 30 (60%) isolates, a MIC of 16 μg/ml for 15 (30%) isolates, a MIC of 8 μg/ml for three (6%) isolates, and a MIC of 4 μg/ml for two (4%) isolates. The distribution of chlorhexidine MICs is shown in Fig. 1. Chlorhexidine had a MIC of 2 μg/ml for XLOLR and *Escherichia coli* ATCC 25922. A chlorhexidine-resistant strain (NTUH-2044; chlorhexidine MIC, 32 μg/ml) and a chlorhexidine-sensitive strain (NTUH-9770; chlorhexidine MIC, 4 μg/ml) were used (1). Details of the characteristics of the bacterial strains and plasmids used in this study are listed in Table 1.

Genomic DNAs from NTUH-2044 were extracted and partially digested with *Sau*3AI (3, 19). Fragments of 3 to 5 kb in length were recovered. Construction of λ-ZAP II libraries and insertion of in vivo excision phages into pBK-CMV phagemids were carried out (Stratagene, La Jolla, Calif.) (3, 20).

DNA sequencing was performed on an ABI PRISM 377 DNA sequencer. Primers (5′-AATTAACCCTCACTAAAGG G-3′ and 5′-GTAATACGACTCACTATAGGGC-3′) based on the phagemid vector were designed for sequencing inserts. Nucleotide sequences and deduced amino acid sequences were analyzed and compared with those listed in GenBank, the SWISS-PROT databases, and the BLAST network service at the National Center for Biotechnology Information.

An integrative vector from pUTKm1Δ*tnp* Δmini-Tn*5* (10), with *E. coli lacZ* in the *Sal*I restriction site and *K. pneumoniae cepA* in the *Eco*RI restriction site, was transformed into *E. coli* ATCC 25922 by both conjugation and electroporation (3, 10).

For knockout experiments, the following procedures were performed. (i) A linear DNA, *cat*::*cepA*, was constructed by inserting a chloramphenicol acetyltransferase cassette (gift from D. E. Taylor) (24) into the *Acc*I site within *cepA*. The DNA fragment containing *cepA* was amplified by PCR using primers PT-F1 (5′-CAACTCCTTCGCCTATCCCG-3′) and PT-R1 (5′-TCAGGTCAGACCAAACGGCG-3′) to anneal sites −72 and +958, respectively. (ii) The *cat*::*cepA* fragment was also inserted into the *Eco*RI site on suicide vector pUTKm1Δ*tnp* Δ*bla* Δmini-Tn*5* (10). (iii) A 200-bp intragenic fragment (*cepA* nucleotides +97 to +297, amplified by PCR) in the same suicide vector carrying a chloramphenicol acetyltransferase cassette was also constructed. Linear DNA (*cat*::*cepA*) and suicide vectors for both constructs were transformed into NTUH-2044 by electroporation and conjugation (3, 10).

Screening of the phagemid expression library in XLOLR revealed 20 clones which grew on plates containing chlorhexidine (16 μg/ml). DNA sequencing of the 20 clones revealed three different inserts. However, all clones contained a 903-nucleotide locus (Fig. 2A). Plasmid carrying this locus was retransformed into XLOLR by 42°C heat shock. Retransformants again grew on plates containing chlorhexidine (16 μg/ml).

Sequencing of this open reading frame (ORF) showed a 90% similarity to *yiip*, a putative transporter in *E. coli* K12 (2). The deduced amino acid sequence showed that it could be a cation efflux pump. Thus, this ORF was designated *cepA* (GenBank accession number AB073019). Proteins displaying similarity to the deduced amino acid sequence of *cepA* included several putative permease proteins in *E. coli* and two putative transmembrane efflux proteins in *Salmonella enterica* serovar Typhi (Fig. 2B). The adjacent ORFs upstream and downstream with respect to *cepA* had nucleotide sequences 87%

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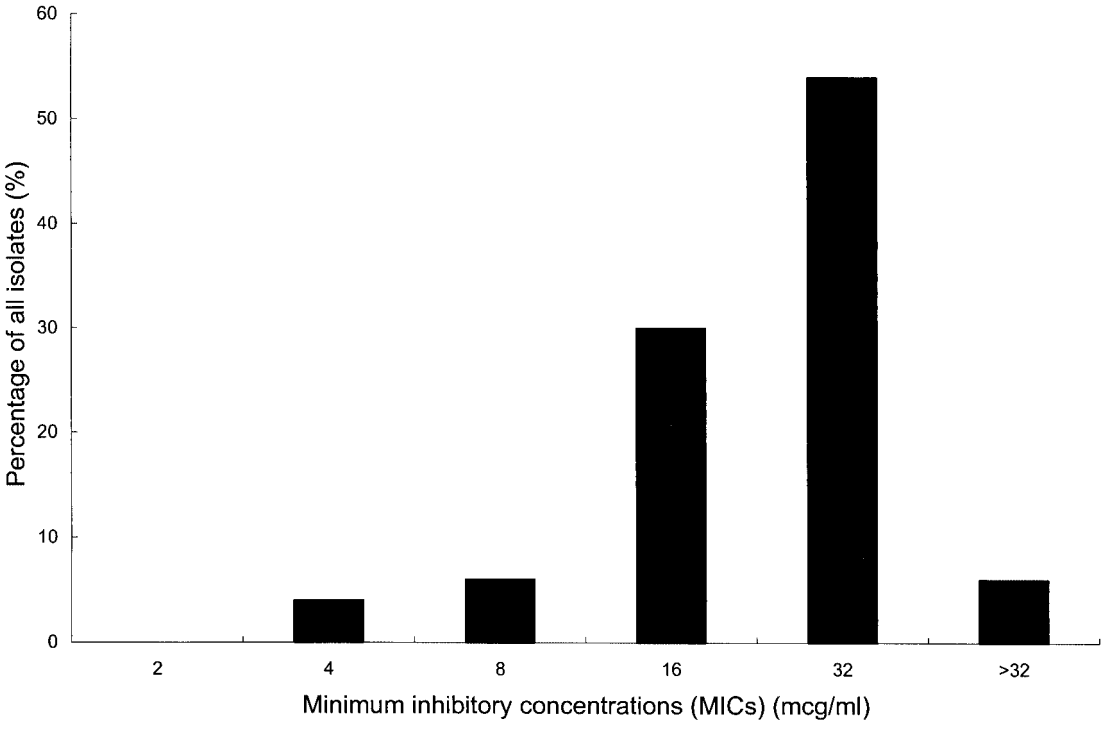

FIG. 1. Distribution of chlorhexidine MICs for 50 randomly selected clinical *K. pneumoniae* isolates.

similar to *E. coli* transcription factor *cpxR* and *Enterobacter cloacae* phosphofructokinase *pfkA*, respectively. The chromosome and plasmid were separated by pulsed field gel electrophoresis. Results of a nested PCR using *cepA*-specific primer pairs showed that *cepA* was present on the chromosome.

pBK-CMV plasmids carrying *cepA* were also transformed into NTUH-9770 by electroporation. Transformants had a fourfold increase in chlorhexidine MICs. Transformation with suicide vector *lacZ-cepA*-pUTKm1 Δ*tnp* Δmini-Tn*5* into ATCC 25922 yielded *cepA* single-integration recombinants (confirmed by PCR with different alignments of primer pairs) for which chlorhexidine showed a twofold increase in MIC.

No chloramphenicol-resistant transformants were obtained by transformation of the linear DNA *cat*::*cepA* or intragenic fragment in a suicide vector. Transformations with suicide plasmid vector *cat*::*cepA*-pUTKm1 Δ*tnp* Δ*bla* Δmini-Tn*5* yielded chloramphenicol-resistant clones. However, PCR amplification with PT-F1 and PT-R1 yielded both 1-kb and 1.8-kb products (not shown), which corresponded to the sizes of wild-type *cepA* and *cat*::*cepA*, respectively. This result suggested that an integration, not a knockout, had occurred.

cepA was expressed in XLOLR transformants carrying *cepA*::pBK-CMV as previously described (19, 20). A pBK-CMV plasmid without *cepA* was used as a control. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis with Coo-

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
E. coli strains		
XLOLR	Recipient of λ-ZAP II vector; chlorhexidine sensitive	Stratagene
S17-1/λ*pir*	Donor of pUTKm1 Δ*tnp* Δ*bla* Δmini-Tn*5* during conjugation; ampicillin sensitive	7
ATCC 25922	Chlorhexidine sensitive and ampicillin sensitive	American Type Culture Collection
Plasmids		
pBK-CMV	Phagemid vector resulting from in vivo excision of λ-ZAP II vector in *E. coli* XLOLR	Stratagene
pUTKm1 Δ*tnp* Δ*bla* Δmini-Tn*5*	Suicide vector constructed from pUTKm1 by deleting *tnp, bla*, and mini-Tn*5* transposon through restriction enzymatic digestion (*Sal*I, *Apa*LI, and *Eco*RI, respectively) followed by ligation	This work
pCRII-TOPO	TA cloning vector	Invitrogen

A

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1     CAACTCCTTC GCCTATCCCG TGAGCGTCAC AAGTTCGGGT TATACTAAGC GCATTGCAGG

                 +1
61    AGAAGGAGCC TATATGAATC AATCTTATGG CCGGTTGGTC AGTCGCGCCG CTATCGCCGC
                 M  N  Q   S  Y  G   R  L  V   S  R  A  A   I  A  A

121   GACGGCTATG GCCTCCGCGT TACTTTTGAT CAAAATTTTT GCGTGGTGGT ATACCGGTTC
       T  A  M   A  S  A  L   L  L  I   K  I  F   A  W  W  Y   T  G  S

181   TGTCAGTATT CTGGCTGCGC TGGTGGATTC GCTGGTGGAC ATTGCCGCCT CGCTGACCAA
       V  S  I   L  A  A  L   V  D  S   L  V  D   I  A  A  S   L  T  N

241   CCTGCTGGTG GTTCGCTATT CGCTACAGCC TGCTGATGAA GAACATACCT TTGGTCATGG
       L  L  V   V  R  Y  S   L  Q  P   A  D  E   E  H  T  F   G  H  G

301   CAAAGCGGAG TCGCTGGCGG CGCTGGCGCA AAGCATGTTT ATCTCCGGCT CGGCGCTGTT
       K  A  E   S  L  A  A   L  A  Q   S  M  F   I  S  G  S   A  L  F

361   CCTGTTTCTC ACCGGCATTC AGCACCTGGT GCGTCCGGAG CCGCTGCAGG CCGCCGGCGT
       L  F  L   T  G  I  Q   H  L  V   R  P  E   P  L  Q  A   A  G  V

421   CGGGGTCGTC GTCACATTGA TCGCCCTCGT TAGTACGCTG GCGCTGGTGA CTTTCCAGCG
       G  V  V   V  T  L  I   A  L  V   S  T  L   A  L  V  T   F  Q  R

481   CTGGGTGGTG CGAAAAACCC AGAGCCAGGC GGTGCGGGCG GATATGCTTC ATTATCAGTC
       W  V  V   R  K  T  Q   S  Q  A   V  R  A   D  M  L  H   Y  Q  S

541   TGATGTTATG ATGAACGGCG CCATTCTGGT GGCGCTGGGC CTATCCTGGT ACGGCTGGCA
       D  V  M   M  N  G  A   I  L  V   A  L  G   L  S  W  Y   G  W  H

601   TCGCGCCGAC GCGTTGTTTG CCCTGGGGAT TGGCATCTAT ATTTTATATA GCGCGCTGCG
       R  A  D   A  L  F  A   L  G  I   G  I  Y   I  L  Y  S   A  L  R

661   GATGGGCTAT GAGGCGGTTC AGTCACTACT CGACCGCGCC TTGCCTGACG AGGAGCGTCA
       M  G  Y   E  A  V  Q   S  L  L   D  R  A   L  P  D  E   E  R  Q

721   GGACATTATC ACCATCGTGA CCGCATGGCC CGGCATCCGC GGGGCGCACG ATCTACGAAC
       D  I  I   T  I  V  T   A  W  P   G  I  R   G  A  H  D   L  R  T

781   GCGGCAGTCA GGGCCGACCC GCTTTATTCA GATTCATTTG GAAATGGAAG ATAACCTCCC
       R  Q  S   G  P  T  R   F  I  Q   I  H  L   E  M  E  D   N  L  P

841   GCTGGTGCAA GCCCACGTGA TTGCAGACCA GGTGGAGCAG GCGATTCTGC GCCGTTTCCC
       L  V  Q   A  H  V  I   A  D  Q   V  E  Q   A  I  L  R   R  F  P

901   GGGGTCCGAT GTCATTATCC ATCAGGATCC CAGCTCTGTG GTGCCAGCGG CGCAGCAGGG
       G  S  D   V  I  I  H   Q  D  P   S  S  V   V  P  A  A   Q  Q  G

961   CTTTTTTGAG CGTTAGGTTA TAGTCTGTAA ACTCGATGTA AAAATGTGGG GCAGATCGGC
       F  F  E   R  .

1021  ATTTTTTGTA TAAATTACCG CCGTTTGGTC TGACCTGA
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FIG. 2. (A) Nucleotide sequences of *cepA* and flanking regions. The predicted promoter region is underlined. The start codon is marked by +1. (B) CepA amino acid sequence alignment with putative transport system permease protein in *E. coli* (accession number NC 002655) and putative transmembrane efflux protein in *Salmonella enterica* serovar Typhi (accession number NC 003198). A dark background indicates identical residue homologies, and the lighter background shows residues with similarities. Alignment was done using Macvector 6.5 sequence analysis software (Oxford Molecular Group). Comparison of the CepA sequences with those of putative cationic efflux pumps in *E. coli* and *S. enterica* serovar Typhi revealed 83 to 84% similarity.

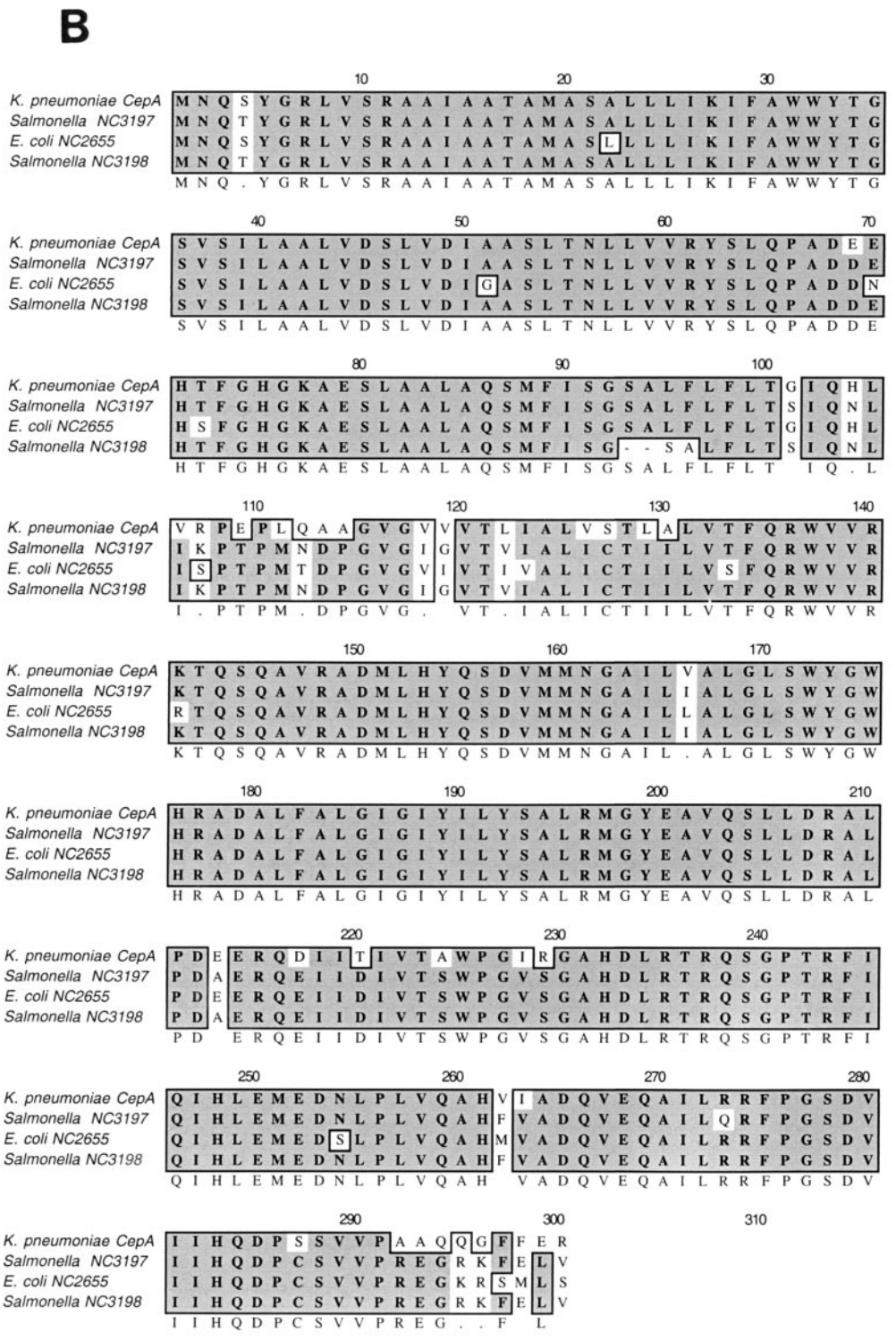

FIG. 2—*Continued.*

massie blue staining revealed a 33-kDa protein (the predicted size of CepA) carrying *cepA*::pBK-CMV in XLOLR but not in the control vector (data not shown).

Chlorhexidine is a cationic biguanide that kills bacteria by membrane damage followed by intracellular coagulation (8). Gram-negative bacteria are less susceptible to chlorhexidine than gram-positive bacteria (12, 18). Impermeability of the outer membrane to chlorhexidine has been implicated in *Pseudomonas*, *Proteus*, and *Providencia* (12, 18). Formation of biofilm prolongs survival of *Serratia* and *Burkholderia* on expo-

sure to chlorhexidine (12, 18). A chlorhexidine-degrading enzyme has been discovered in *Achromobacter xylosoxidans* (14).

We demonstrated that transformation of *cepA*-containing phagemid into XLOLR resulted in significant elevation of chlorhexidine MICs. A single integration of *cepA* was sufficient to increase chlorhexidine MICs for *E. coli* twofold. Transformation of *cepA*-containing pBK-CMV into NTUH-9770 also resulted in a fourfold elevation of chlorhexidine MICs. These results suggest that *cepA* is associated with chlorhexidine resistance. Attempts in three different experiments failed to obtain knockout mutants. Therefore, *cepA* is probably essential. Our findings indicate that drug efflux might be an important mechanism of chlorhexidine resistance in *K. pneumoniae* and possibly in other related gram-negative bacteria. A similar mechanism is observed in staphylococci, in which export proteins, encoded by *qacA* and/or *qacB*, are also responsible for chlorhexidine resistance (17, 22).

Chlorhexidine is usually supplied in a 0.5 to 4% solution for handwashing (9). In a clinical environment, chlorhexidine concentrations 10- to 50-fold higher than MICs are required to produce 99.99% killing (4 $\log_{10}$ reduction) within 10 min at 20°C (8). Handwashing with chlorhexidine has failed to control nosocomial spread of methicillin-resistant *Staphylococcus aureus* with *qacA* and/or *qacB*-mediated resistance (MIC, 2 to 4 μg/ml) (16). Because of the higher levels of MICs required, chlorhexidine-resistant *K. pneumoniae* could be clinically more problematic than staphylococci.

In conclusion, *cepA* is associated with chlorhexidine resistance in *K. pneumoniae*. CepA protein may act as a cation efflux pump.

Nucleotide sequence accession number. The sequence for ORF *cepA* has been listed in GenBank with the accession number AB073019.

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