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KlcA$_{HS}$ genes are ubiquitous in clinical, *bla*$_{KPC-2}$-positive, *Klebsiella pneumoniae* isolates☆

Wei Liang[a], Yu Tang[b], Gang Li[b], Pinghua Shen[b], Yueru Tian[b], Haoqin Jiang[b], Xiaofei Jiang[b,*], Yuan Lu[b,*]

[a] Department of Laboratory Medicine, the Second People's Hospital of Lianyungang City, Jiangsu Province, China
[b] Department of Laboratory Medicine, Huashan Hospital, Shanghai Medical College, Fudan University, Shanghai, China

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ABSTRACT

Carbapenemase-producing *Klebsiella pneumoniae* has emerged and spread widely throughout the world. The mechanisms involved remain unclear. To provide insight, five plasmids were obtained from carbapenemase-producing *K. pneumoniae* clinical isolates. The five sequences were acquired, aligned and analyzed. In addition to the *bla*$_{KPC-2}$ gene, which encodes beta lactamase, essentially all the plasmids contained a putative anti-restriction protein-encoding gene, *KlcA*$_{HS}$. The *KlcA*$_{HS}$ gene was found in 98.2% of the *bla*$_{KPC-2}$-positive, imipenem-resistant *K. pneumoniae* clinical isolates and in < 1% of the *bla*$_{KPC-2}$-negative control group. A searched of the GenBank database indicated that KlcA$_{HS}$ was mainly submitted by Chinese investigators beginning in 2010. Seventeen different KlcA amino acid sequences were found in the database using the restricting words: KlcA and *Klebsiella pneumoniae*. These sequences were used to generate a phylogenetic tree via MEGA6 software, revealing a distant evolutionary relationship between KlcA$_{HS}$ and other KlcAs. The secondary structure of KlcA$_{HS}$, predicted with PROMALS3D software, exhibited highly conserved α-helices and β-strands. KlcA$_{HS}$ expressed anti-restriction activity in vivo.

In summary, *KlcA*$_{HS}$ genes are ubiquitous in *bla*$_{KPC-2}$-positive *Klebsiella pneumoniae* clinical isolates collected at Huashan Hospital, China. The KlcA$_{HS}$ protein possesses a secondary structure similar to that exhibited by anti-restriction proteins and displays anti-restriction activity. As such, KlcA$_{HS}$ is a probable factor in the accelerated spread of *bla*$_{KPC-2}$ and carbapenem-resistance among clinical, *K. pneumoniae* isolates.

1. Introduction

Antibiotic resistance is an increasing problem that limits the availability of antibiotics to treat bacterial infections. The global speed with which carbapenem-resistance is spreading among *K. pneumoniae* is accelerating; the development of new strategies to restrict this spread is urgent. Bacterial strains can generate antibiotic resistance phenotype by intrinsic and extrinsic factors, such as mutation, genomic recombination of foreign DNA into the chromosome and horizontal gene transfer (HGT) (Davies and Davies, 2010). Usually, many factors are involved. HGT enriches the gene pool and promotes the probability of evolution. Genes are often retained selectively when they provide advantages to host strains (Brown-Jaque et al., 2015). Virulence factors, biofilm and antibiotic resistance, for example, increase the survival of certain strains in hostile environment (Mathers et al., 2015).

The horizontal transfer of plasmids harboring resistance genes is an important mechanism in the spread of antimicrobial resistance (Carattoli, 2013). Gene transfer, however, is limited by bacterial Restriction-Modification (RM) systems, which function as prokaryotic immune systems that attack and degrade foreign DNA entering the cell (Bertani and Weigle, 1953). The RM systems are classified into four types, I-IV, dependent upon the complexity of their structure and function. RM system type I is the most complex exhibiting both restriction endonuclease and modification methyltransferase activities in one large complex composed of three subunits encoded by the *hsdR*, *hsdM* and *hsdS* genes where *hsd* denotes 'host specificity of DNA' (Tock and Dryden, 2005). The Type I RM system is comprised of two opposing activities: restriction endonuclease (REase), which recognizes and cleaves a specific DNA sequence, and cognate methyltransferase (MTase), which methylates adenine or cytosine bases within the same

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* Corresponding authors.
E-mail addresses: jiangxi2154@aliyun.com (X. Jiang), luyuan@sccl.org.cn (Y. Lu).

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recognition sequence and, thus, protects the site from cleavage (Loenen et al., 2014). Some plasmids, however, encode anti-restriction proteins that enable them to evade host restriction (Delver et al., 1991).

The aim of the present study was to identify and delineate the putative gene, *KlcA*$_{HS}$, located in plasmids extracted from clinical isolates of *K. pneumoniae*. The gene product, KlcA$_{HS}$, exhibits 43% identity and 66% similarity to KlcA$_{136}$ derived from pBP136 (Kamachi et al., 2006), and demonstrates anti-restriction activity suggesting that it facilitates HGT.

2. Materials and methods

2.1. Collection of Klebsiella pneumoniae clinical isolates

Two hundred twenty-four clinical, *K. pneumoniae* isolates used in this study were collected between 2006 and 2010 at Huashan Hospital, Shanghai, China. One hundred twelve of these isolates (the test group) exhibited both imipenem-resistance, confirmed by the Kirby–Bauer disk diffusion method according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2012), and the *bla*$_{KPC-2}$ gene verified using PCR. The other 112 samples, composing as the control group, were imipenem-sensitive and *bla*$_{KPC-2}$-negative. The two groups of isolates came from similar clinical backgrounds, both were collected at the same time in the same wards. Five plasmids: pHS062105, pHS082416, pHS092753, pHS092839 and pHS10505, were obtained from isolates belonging to the test group.

2.2. Plasmid sequence assembly and analysis

Plasmid pHS062105 was isolated by the alkaline lysis method (Qiagen GmbH, Germany). Purified pHS062105 was sequenced completely, annotated and deposited in GenBank (Accession: NC_023331) according to protocols described previously in detail by us (Li et al., 2014). Briefly, a fragment library was generated from 1 μg pHS062105 using the Ion Plus fragment library kit (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA). DNA sequencing and sequence assembly were performed using the Ion Torrent platform (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA). Gaps were closed by sequencing amplicons generated by primer-walking PCR performed on linking clones. Open reading frames were identified and annotated by searching against the NCBI nonredundant protein database. The remaining four plasmids (pHS082416, pHS092753, pHS092839 and pHS10505), isolated at different times over the course of the collection period (2006–2010), were sequenced partially.

2.3. Genetic map of neighboring sequence of bla$_{KPC-2}$ gene

Preliminary screening results suggested that pHS082416, pHS092753, pHS092839 and pHS10505 had a *bla*$_{KPC-2}$ neighboring structure similar to that found in pKP048 (Shen et al., 2009). The PCR mapping method was employed to sequence the specific region nearby the *bla*$_{KPC-2}$ gene of the plasmids cited immediately above using the approach described by Li et al. (Li et al., 2014). A set of primers with a uniform annealing temperature of ~55 °C was designed to generate overlapping PCR fragments covering the target region (Table 1). All the amplicons generated were sequenced. The nucleotide sequences of five sequenced plasmids were aligned using software BRIG Version 0.95 (Alikhan et al., 2011) (http://sourceforge.net/projects/brig/).

2.4. KlcA$_{HS}$ and ST distribution

Multilocus Sequence Typing (MLST), based upon nucleotide variations in the following seven housekeeping genes: *gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB*, was conducted for all isolates in accordance with methods described previously (Diancourt et al., 2005). These genes were amplified, sequenced, and aligned in the MLST databases (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html) using software available online (https://online.phyloviz.net/index). The result of MLST (ST) was assigned to each clinical isolate. Presence of the *KlcA*$_{HS}$ gene was screened among all isolates by PCR; the primers were designed with Primer Premier 6 software (PREMIER Biosoft International, Palo Alto, CA, USA).

2.5. KlcA proteins, evolutionary relationships, physical and chemical parameters

The amino acid sequences of the proteins encoded by *KlcA*$_{HS}$ were searched in the GenBank database with the restricting words: KlcA and *Klebsiella pneumoniae*. Redundant and repeat sequences were removed online (http://web.expasy.org/decrease_redundancy/). The remaining variations of the KlcA amino acid sequence were collected, and their physical and chemical parameters were predicted online using the ProtParam tool (http://web.expasy.org/protparam/). All protein concentrations are given for the monomeric form. Molecular weights of the monomers, which include the N-terminal methionine, are shown in Supplemental Table I. Both the KlcA amino acid sequences reported previously and those selected in the present study were analyzed using MEGA6 software (www.megasoftware.net) to determine their evolutionary relationships and construct a phylogenetic tree (Tamura et al., 2013). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The phylogenetic tree is drawn to scale; each branch is equivalent in length to the evolutionary distance used to construct the tree.

2.6. Conserved, secondary KlcA$_{HS}$ structure

Since ArdB and KlcA exhibit similar anti-restriction activities, the ArdB and KlcA amino acid sequences reported in GenBank were downloaded. These sequences, together with the sequence for KlcA$_{HS}$, were aligned and the conserved secondary structure was predict with PROMALS3D software (Pei et al., 2008) (http://prodata.swmed.edu/PROMALS3D).

2.7. KlcA$_{HS}$ anti-restriction activity in vivo

The following experiment was conducted in accordance with methods previously described (Serfiotis-Mitsa et al., 2010). *Escherichia coli* DH5α was selected as a cloning strain. *E. coli* strains, NM1261(r^-m^-, restriction endonuclease-negative, methyltransferase-negative) and NM1041 (hsd^+clpX^-) were gifts obtained from Professor David T. F. Dryden (EaStChem School of Chemistry, University of Edinburgh, UK) and used to assess the anti-restriction activity of KlcA$_{HS}$ in vivo. To evaluate the anti-restriction activity expressed by KlcA$_{HS}$, *KlcA*$_{HS}$ gene was cloned from pHS10505 with predesigned primers for the NdeI and EcoRI recognition sequences (Table 1). The gene was then ligated into the kanamycin-resistant expression vector, pET24a, at the NdeI and EcoRI sites. DNA sequencing in both directions with pET24aF and R primers shown in Table 1 evidenced the gene was inserted in the correct direction. Competent, $CaCl_2$-treated *E. coli* NM1261(r^-m^-) and NM1041 (hsd^+clpX^-) cells were transformed with recombinant (pET24a-KlcA$_{HS}$) and wild-type (pET24a) plasmids, respectively. The transformants were spread on LB agar plates containing 25 μg/ml kanamycin with or without 80 μg/ml 2-aminopurine (2-AP), and the growth of each transformant was determined.

3. Results

3.1. Plasmid sequences

The complete nucleotide sequence of pHS062105 and four partial sequences of pHS082416, pHS092753, pHS092839 and pHS10505 were deposited in the GenBank database under Accession numbers: NC_

Table 1
Primers used.

Primer name	Sequence (5′-3′)	Annealing temperature (°C)	Product (bp)
KPC-2F	TGTAAGTTACCGCGCTGAGG		
KPC-2R	CCAGACGACGGCATAGTCAT	55	584
$KlcA_{HS}$F	GGCTTATTGGCTTATGTGG		
$KlcA_{HS}$R	GTAGAGGCAAGCGGTAAT	55	145
pET24a-F	TAATACGACTCACTATAGG		
pET24a-R	GCTAGTTATTGCTCAGCGG	55	258
KlcAF	ACGGTGTCATATGATGCAAACAGAACTTAA		
KlcAR	GCTAGAATTCCTAGTCTATTGCGGCCAAG	50	426

Forward (F) and reverse (R) primers used to detect: $bla_{KPC\text{-}2}$ (KPC-2F/R), $KlcA_{HS}$, ($KlcA_{HS}$F/R), and the kanamycin-resistant expression vector, pET24a (pET24aF/R). KlcAF/R was used to clone the entire $KlcA_{HS}$ gene. The NdeI and EcoRI enzyme recognition sequences are underlined.

023331, KF724507, KF826293, KF724506 and KF826292, respectively. The five sequences are shown in ring form with pHS10505 as the reference (Supplemental Figure 1).

3.2. $KlcA_{HS}$ gene and ST distribution

$KlcA_{HS}$ was found much more often in the test, relative to the control group (98.2% vs. < 1%). The distribution of STs among these two groups was also significantly different (Fig. 1).

3.3. Evolutionary relationships, and physical and chemical parameters of KlcA

Ninety-eight KlcA amino acid sequences were found in a GenBank search; 17 of these remained after the redundant and repeat sequences were eliminated. The physical and chemical parameters of each of these KlcAs are shown in Supplemental Table I. A phylogenetic tree was constructed from these, as well as reported, KlcA sequences to establish their evolutionary relationships (Fig. 2). $KlcA_{HS}$ was assigned to branch no. 16, which contains 20 records. Records pertaining to $KlcA_{HS}$ were principally submitted by Chinese investigators beginning in 2010 (Fig. 3).

3.4. Predicting the conserved, secondary structure of $KlcA_{HS}$

Anti-restriction proteins, KlcA_RK2 and ArdB_pKM101, were selected as archetypal sequences to perform the alignment of the $KlcA_{HS}$ sequence studied here and anti-restriction sequences reported previously (Belogurov et al., 1993; Larsen and Figurski, 1994). The alignment shows that $KlcA_{HS}$ exhibits highly conserved α-helices and β-

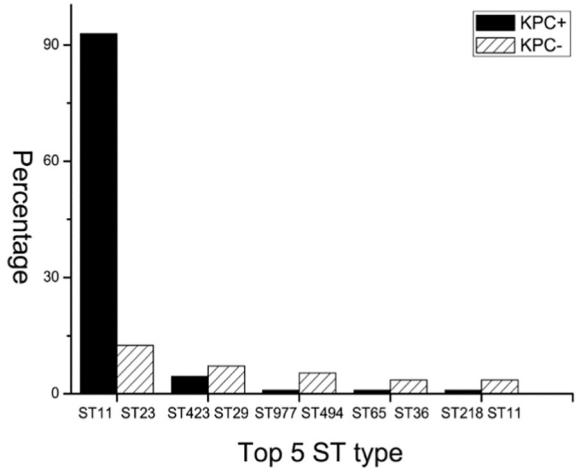

Fig. 1. The top 5 STs of clinical isolates obtained in the test and the control groups. The distribution of STs is scattered in the control group, but a centralized trend is observed in the test group.

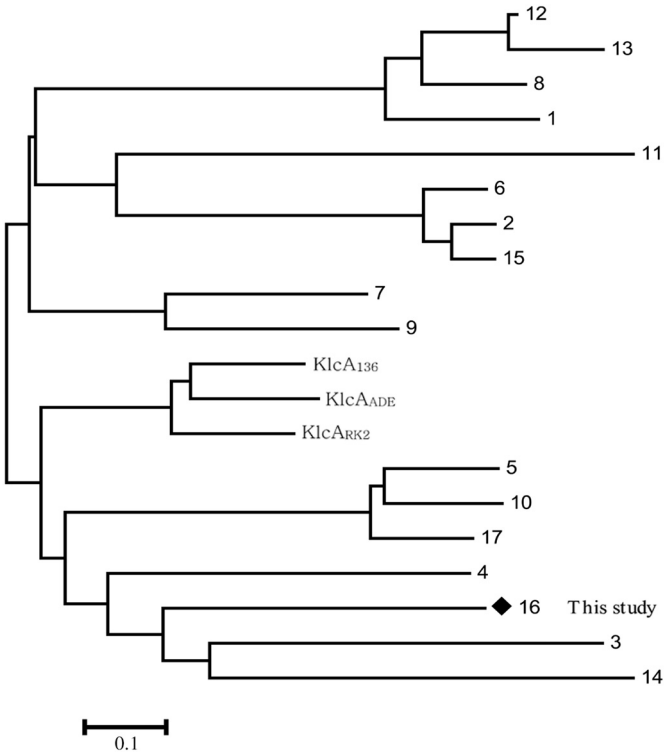

Fig. 2. Evolutionary tree was generated automatically with MEGA6 software (Tamura et al., 2013). $KlcA_{HS}$ (number 16) forms a branch in the evolutionary tree that is distant from other KlcAs and the reported KlcA.

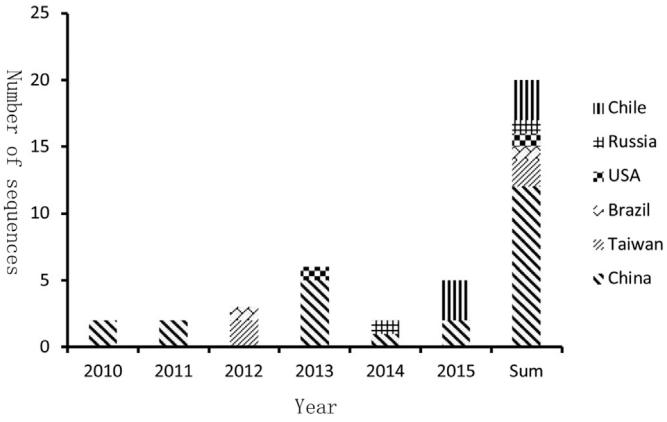

Fig. 3. Submission time and worldwide distribution of $KlcA_{HS}$. X and Y axes denote the year and number of $KlcA_{HS}$ sequences submitted, respectively.

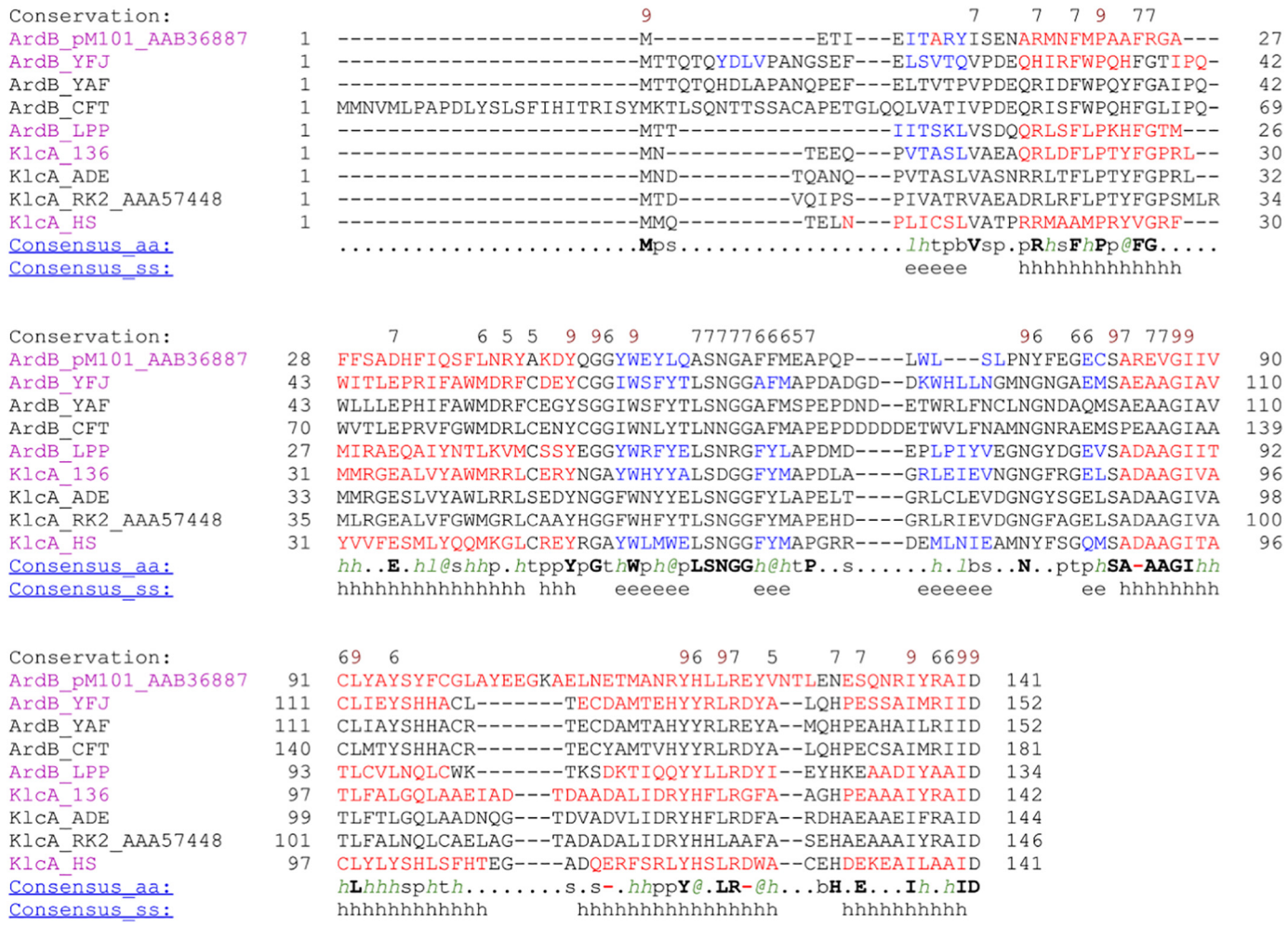

Fig. 4. Anti-restriction proteins, KlcA_RK2 and ArdB_pKM101, were selected as archetypal sequences to perform the alignment (Belogurov et al., 1993; Larsen and Figurski, 1994). A conservation index > 4 is indicated on the first line. Consensus_aa (consensus amino acid sequence) and Consensus_ss (consensus predicted secondary structures) are shown on the last two lines. Representative sequences are colored according to predicted secondary structures: α-helix (red) and β-strand (blue). The symbols *h* and *e* are shown in the Consensus_ss line to indicate α-helix and β-strand, respectively. The annotations marked in the Consensus_aa line can be interpreted according to the literature (Serfiotis-Mitsa et al., 2010). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

strands, while the loops vary in length and amino acid composition (Fig. 4).

3.5. $KlcA_{HS}$ displays anti-restriction activity in vivo

The growth of *E. coli* NM1041 (hsd^+clpX^-) harboring pET24a alone was poor, and nearly inhibited completely on LB agar plate supplemented with kanamycin (25 μg/ml) and 2-AP (80 μg/ml). In contrast, NM1041 carrying recombinant plasmid pET24a-$KlcA_{HS}$ grew well on the same agar plate (Fig. 5). This finding suggests that expression of the $KlcA_{HS}$ gene confers anti-restriction activity which disrupts the RM system (EcoKI) of the host strain. Although the growth of *E. coli* NM1041 harboring pET24a was restricted, *E. coli* NM1261 (r^-m^-) harboring pET24a grew well on LB agar plates containing kanamycin (25 μg/ml) and 2-AP (80 μg/ml).

4. Discussion

The worldwide spread of *Klebsiella pneumoniae* carbapenemase-producing bacteria is a significant challenge to workers in the medical field. It has narrowed the choice of antibiotics and contributed to the increase in patient morbidity and mortality (Schwaber et al., 2006). Consequently, carbapenem-resistance is a huge social burden (Gilbert et al., 2010). The exact mechanisms by which drug-resistance genes are disseminated among bacteria are obscure. It is clear, however, that the horizontal transfer of plasmids containing resistance genes is essential for dissemination (Carattoli, 2013). The mobility of plasmid-harboring genes responsible for drug resistance is considered an important reason for the recent and rapid global increase in antimicrobial resistance (Nordmann et al., 2011). Genetic transfer occurs via three well known mechanisms: conjugation, transformation and transduction (Brown-Jaque et al., 2015). Plasmid mobility is affected by bacterial RM systems, which function as prokaryotic immune systems that attack foreign DNA entering the cell (Bertani and Weigle, 1953).

RM systems, which are widespread in eubacteria and archaea as well as bacteria (Roberts et al., 2015), can damage foreign DNA on mobile genetic elements during HGT. RM systems, however, are not a permanent and perfect defense against invading foreign DNA; many plasmids employ effective approaches to escape restriction (Wilkins, 2002). Conjugative plasmids and transposons, which contain *ardA*, *ardB*, *ardC* and *ardD* genes, encode anti-restriction proteins that allow them to avoid restriction. The ArdA, ArdB, ArdC and ArdD proteins inhibit Type I RM enzymes (Balabanov et al., 2012; Belogurov et al., 2000; Belogurov et al., 1993; Delver et al., 1991; Serfiotis-Mitsa et al., 2010). The ArdA protein, for example, carries a considerable negative charge on the surface, has a spatial structure like double helical DNA,

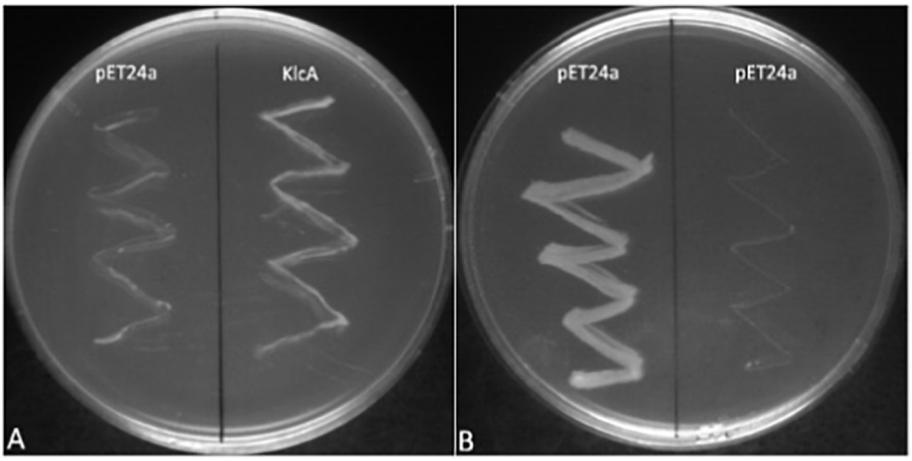

Fig. 5. Anti-restriction activity of $KlcA_{HS}$. Half plates inoculated with the host strain carrying the indicated plasmid were incubated for 12 h at 37 °C, then growth was assessed. (A) NM1041 (hsd^+clpX^-) harboring pET24a (left) versus NM1041 harboring pET24a-$klcA_{HS}$ (right). (B) NM1261(r^-m^-) carrying pET24a (left) versus NM1041 carrying pET24a (right). A representative experiment of three experiments conducted with different colonies is shown.

and exhibits a high binding affinity for RM enzymes. As such, ArdA exerts anti-restriction activity by virtue of its ability to bind RM enzymes (McMahon et al., 2009; Serfiotis-Mitsa et al., 2010). The mechanisms by which ArdB, ArdC and ArdD proteins inhibit Type I RM enzymes are presently unclear.

KlcA was first gene identified that constitutes the *kilC* operon found in IncP plasmids (Larsen and Figurski, 1994). KlcA exhibits significant homology with the anti-restriction ArdB protein (Kamachi et al., 2006). KlcA from plasmid pBP136 shows 30% identity and 46% similarity to ArdB encoded by plasmid pKM101, while KlcA from plasmid pRK2 shows 31% identity and 56% similarity (Belogurov et al., 1993). Likewise, we found here that $KlcA_{HS}$ exhibited 30% identity and 56% similarity to ArdB from pKM101. $KlcA_{HS}$ is a small, acidic protein (isoelectric point = 5.52), which is similar in size (16.4 kDa) to ArdB suggesting the likelihood that $KlcA_{HS}$ is an anti-restriction protein. Dryden et al. confirmed that proteins homologous to ArdB, i.e., $KlcA_{ADE}$ and $KlcA_{136}$, acted as anti-restriction proteins and inhibited the four main families of Type I RM systems in vivo (Serfiotis-Mitsa et al., 2010).

Here, we report that *$KlcA_{HS}$* genes are ubiquitous in carbapenemase-producing and bla_{KPC-2} positive clinical *K. pneumoniae* isolates; none were detected in the control group. This finding suggests that *$KlcA_{HS}$* is closely linked to the bla_{KPC-2} gene in *K. pneumoniae* isolates, at least in Huashan Hospital Shanghai, China. It is predicted that $KlcA_{HS}$ has a secondary structure like known anti-restriction proteins, KlcA and ArdB. In this regard, the anti-restriction activity of $KlcA_{HS}$ was assessed using the *E. coli* NM1041 (hsd^+clpX^-) strain, which barely grew on agar plates that contained 2-AP unless the EcoKI RM system was inactivated by anti-restriction proteins (Serfiotis-Mitsa et al., 2010). Our results showed that the growth of NM1041 strains carrying recombinant plasmid pET24a-$KlcA_{HS}$ on 2-AP-containing LB agar plates was significantly greater than the growth of NM1041 strains carrying plasmid pET24a alone. Therefore, it is speculated that the growth of RM system-deficient strains is not affected by the presence of 2-AP. Rather, growth inhibition of *E. coli* NM1041 (hsd^+clpX^-) harboring pET24a is dependent upon the RM system. It can be conjectured that $KlcA_{HS}$ possesses the anti-restriction activity that inhibits the RM system (EcoKI) of the host strain; the precise mechanism, however, is presently unclear.

$KlcA_{HS}$ exist in countries other than China, e.g., Taiwan, Chile, USA and Russia. A blast search of the *$KlcA_{HS}$* sequence in GenBank obtained 100 hits (Gammaproteobacteria); 91 of these were enterobacteria, only 55 were *Klebsiella pneumoniae*. Taken together, these findings indicate that *$KlcA_{HS}$* is distributed worldwide and present in a number of species in addition to *Klebsiella pneumoniae*. As such, $KlcA_{HS}$ may be an important factor in the spread of bla_{KPC-2} between bacteria of various genera and species.

The dissemination of drug-resistance genes involves both horizontal and clonal spread (Chen et al., 2014). Our results demonstrate a sharp contrast in the distribution of ST clones among test and control groups. ST11 dominates the test group accounting for 92.9% of the clones found, a finding consistent with a previous study (Qi et al., 2011). By comparison, the ST clones are scattered throughout the control group where ST23 is found most often.

The mechanisms underlying the principal association of KPC with a single ST11 remains unclear. Whether horizontal or clonal spread dominates the dissemination of drug-resistance genes is equally uncertain. Regardless, the likelihood that $KlcA_{HS}$ plays an important role in the dissemination of carbapenem–resistant *K. pneumoniae* isolates is a reasonable conjecture.

In summary, *$KlcA_{HS}$* genes, which are ubiquitous in clinical, bla_{KPC-2}-positive *K. pneumoniae* isolates, express anti-restriction activity. Expression of these genes facilitates the mobility of plasmids, especially among more compatible clones, e.g., ST11 *K. pneumoniae*. The results of this study broaden the understanding of the critical role of HGT in the spread of antibiotic resistance among pathogenic bacteria.

Conflict of interest statement

None of the co-authors has a conflict of interest to disclose.

Author contributions

Professors Jiang and Lu conceived and designed the study. Wei Liang performed the experiments and wrote the paper. Yu Tang, Gang Li, Pinghua Shen, Yueru Tian, Haoqin Jiang provided suggestions and helped perform the experiments. All authors have read and approved this manuscript.

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Dr. Stephen H. Gregory (Providence, Rhode Island, USA) helped write and edit this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.02.021.

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