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ICE*Apl*Chn1, a novel SXT/R391 integrative conjugative element (ICE), carrying multiple antibiotic resistance genes in *Actinobacillus pleuropneumoniae*

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ABSTRACT

SXT/R391 integrative conjugative elements (ICEs) are capable of self-transfer by conjugation and highly prevalent in various aquatic bacteria and *Proteus* species. In the present study, a novel SXT/R391 ICE, named ICE*Apl*Chn1, was identified in the multidrug resistant (MDR) *Actinobacillus pleuropneumoniae* strain app6. ICE*Apl*Chn1 was composed of the typical SXT/R391 backbone and insertion DNA at eight hotspots, including HS1, HS2, HS3, HS4, HS5, VRII, VRIII and a new variation region VRVI. Many of the insertion contents were not present in other reported SXT/R391 family members, including ICE*Apl*2, a recently identified SXT/R391 ICE from a clinical isolate of *A. pleuropneumoniae*. Remarkably, the VRIII region had accumulated seven resistance genes *tet*(A), *erm*(42), *floR*, *aphA6*, *strB* (two copies), *strA* and *sul2*. Of them, *erm*(42) and *aphA6* emerged for the first time not only in the SXT/R391 elements but also in *A. pleuropneumoniae*. Phylogenetic analysis showed considerable variation of the backbone sequence of ICE*Apl*Chn1, as compared to those of other SXT/R391 ICEs. A circular intermediate form of ICE*Apl*Chn1 was detected by nested PCR. However, the conjugation experiments using different bacteria as recipients failed. These findings demonstrated that SXT/R391 ICEs are able to adapt to a broader range of host bacterial species. The presence of the MDR gene cluster in ICE*Apl*Chn1 underlines that SXT/R391 ICE could serve as an important vector for the accumulation of antibiotic resistance genes.

1. Introduction

Actinobacillus pleuropneumoniae, which is the etiological agent of porcine contagious pleuropneumonia, is one of the most significant pathogens of pigs and causes considerable economic losses to swine industry worldwide. Due to the serovar diversity (16 serovars) and variations in regional prevalence, there is presently no vaccine with satisfactory protection to control *A. pleuropneumoniae* infection (Sassu et al., 2017), thus antibiotic use is still the most effective measure for the treatment and prevention of pig pleuropneumonia. However, increased resistance to the commonly used antibiotics has been reported worldwide (Gutiérrez-Martín et al., 2006; Vanni et al., 2012) and most of the identified resistance genes were located on relatively small plasmids in *A. pleuropneumoniae* (Juteau et al., 1991; Ito et al., 2004; Blanco et al., 2006; Bossé et al., 2015).

Integrative and conjugative elements (ICEs) are mobile genetic elements that are important contributors to horizontal gene transfer and provide useful properties for the bacterial host to adapt to particular environmental conditions. This kind of element can be self-excised from the host chromosome, transfer by conjugation in an intermediate circle form and site-specifically insert and replicate within the new host chromosome (Burrus et al., 2006; Wozniak and Waldor, 2010). Related ICE can be grouped, and members of the SXT/R391 family, defined by the presence of a conserved integrase (Int) and site-specific recombination into the chromosome within the *prfC* gene, are amongst the most well studied (Wozniak et al., 2009). All identified SXT/R391 ICEs have large sizes ranging from approximately 79kb to 110kb and share similar genetic structures. A set of 52 conserved core genes serve as the backbones of SXT/R391 ICEs and about half are essential for basic functions, including integration/excision, conjugative transfer, and regulation (Ceccarelli et al., 2008; Wozniak et al., 2009; Poulin-Laprade and Burrus, 2015; Poulin-Laprade et al., 2015). Within the conserved backbone, insertions in variable regions (VRI-V) and “hotspots” (HS1-5) have been shown to contain a diversity of genes associated with antibiotic and heavy metal resistance, toxin-antitoxin systems, restriction modification systems and alternative metabolic

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pathways (Wozniak and Waldor, 2009; Wozniak et al., 2009; Bordeleau et al., 2010; Rodríguez-Blanco et al., 2012).

The SXT/R391 ICEs are highly prevalent in *Vibrio* species and also present in a wide range of bacteria from aquatic environments (Wozniak et al., 2009; Rodríguez-Blanco et al., 2012; Wang et al., 2016). This kind of ICEs is the main contributor to the acquisition of multidrug resistance in the seventh pandemic lineage of *V. cholerae*, which is the causative agent of cholera (Spagnoletti et al., 2014). Recent studies have shown that *Proteus mirabilis* is another important host bacterium of SXT/R391 ICEs. SXT/R391 elements have been identified in *P. mirabilis* isolates from human, gull, chicken and swine, and play an important role in the dissemination of the cephalosporinase gene bla_{CMY-2} (Harada et al., 2010; Aberkane et al., 2016; Lei et al., 2016). Recently, a SXT/R391 ICE named ICE*Apl*2 carrying four antimicrobial resistance genes *flo*R, *str*AB, *sul2* and *dfr*A1 was identified in serovar 8 *A. pleuropneumoniae* strain MIDG3553 in UK (Li et al., 2018). In this study, we report a second distinct member of the SXT/R391 family, named ICE*Apl*Chn1, recovered from a Chinese serovar 5 isolate of this important swine pathogen, and characterize the genetic structure of this element.

2. Materials and methods

2.1. Bacterial strain and susceptibility testing

A. pleuropneumoniae strain app6 was isolated in October 2013 from a case of porcine respiratory infection at a farm in Shanghai, China. Serotyping was determined by a multiplex PCR (Bossé et al., 2014). Antimicrobial susceptibility testing was performed by broth microdilution according to the recommendations of the CLSI documents VET01-A4 (Clinical and Laboratory Standards Institute [CLSI], 2013). The following 17 antimicrobial agents were tested: phenicols (florfenicol and chloramphenicol), tetracyclines (tetracycline), aminoglycosides/aminocyclitols (gentamicin, amikacin, kanamycin and streptomycin), macrolides (tilmicosin and erythromycin), lincosimides (clindamycin and lincomycin), folate pathway inhibitors (sulfamethoxazole), penicillins (ampicillin and penicillin), cephalosporins (ceftiofur), fluoroquinolones (enrofloxacin) and pleuromutilins (tiamulin). *A. pleuropneumoniae* ATCC 27090 served as the quality control strain.

2.2. Determination of the ICEAplChn1 sequence and annotation

Genomic DNA of *A. pleuropneumoniae* strain app6 was prepared using the Wizard Genomic DNA Purification Kit (Promega, Beijing, China) according to the manufacturer's instructions. Whole genome sequencing was performed by using the Illumina Miseq platform with a 300-bp paired-end library and an average coverage of about 200-fold average coverage (Majorbio, Shanghai, China). De novo assembly was performed using the CLC Genomics Workbench 5 (CLC Bio, Aarhus, Denmark). Antibiotic resistance determinants were detected with the programs ResFinder (Zankari et al., 2012). Gaps between contigs were filled using conventional and primer walking PCR with amplicon sequencing. Annotation of the complete ICE sequence was conducted with RAST (Aziz et al., 2008) and the results were manually checked through comparison with the NCBI database (https://www.ncbi.nlm.nih.gov/). Comparative analysis was carried out with the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3. Phylogenetic analysis of the SXT/R391 ICEs

To explore the evolutionary origin of ICE*Apl*Chn1, phylogenetic analysis was performed based on the backbone sequences built by concatenating the following core genes: *xis*, *int*, *srpRM*, *rumA*, *traID*, *traLEKBVA*, *traC/trhF/traWUN*, *bet*, *exo*, *traFHG*, *eex*, *setCD*, *croS* and *setR*. ICE*Apl*Chn1 and other 14 reference SXT/R391 ICEs (ICE*Apl*2, ICE*Pal*ban1, ICE*Pda*Spa1, ICE*Pmi*Chn1, ICE*Pmi*USA1, ICE*Val*E0601, ICE*Val*HN492, ICE*Vch*ban5, ICE*Vch*Chn2255, ICE*Vch*ind4, ICE*Vch*ind5, ICE*Vch*mex1, R391 and SXTMO10) were subjected to the analysis. In addition, separate phylogenetic analysis was performed for six core genes: *xis*, *int*, *traLEKBVA*, *bet*, *exo*, and *setR*. Phylogenetic trees were constructed with MEGA7 software by maximum-likelihood method (Kumar et al., 2016). Bootstrap values were calculated with 1000 replications.

2.4. Detection of excision and circularization of ICEAplChn1 and conjugation experiments

Detection of the circular intermediate of ICE*Apl*Chn1 was performed using a nested PCR strategy first with the primer set P1 (5′-TTCGCCA GAGCGTCGTAT-3′)/P2 (5′-AGTCAATGGCACGGCGGAGAT-3′) followed by PCR with the nested primer set P3 (5′-ACACTTTCCGAGGTT ACGC-3′)/P4 (5′-GCGTGAAGTCACTGGGAAAGC-3′). The amplicon sequence was obtained by Sanger sequencing and subjected to alignment with the attL and attR of ICE*Apl*Chn1. The mobility of ICE*Apl*Chn1 was tested by conjugation experiments with different rifampicin-resistant bacteria strains as recipients, including *Escherichia coli* EC600, *A. pleuropneumoniae* app10R30 (serovar 7) and *Haemophilus parasuis* D20R30. The latter two strains were derived from two clinical isolates and rifampicin resistance was achieved by serial passages on chocolate-agar plates with increasing rifampicin concentrations (1–50 mg/L). For the mating assay, overnight cultures of the donor and recipient cells were mixed at a ratio of 1:1. The cell mixtures were concentrated by centrifugation, then resuspended and spotted on chocolate or LB agar plates with 6 h incubation at 37 °C. The exconjugants were selected on the chocolate agar (200 mg/L erythromycin and 30 mg/L rifampicin for recipients *A. pleuropneumoniae* app10R30 and *H. parasuis* D20R30) or LB agar (20 mg/L florfenicol and 200 mg/L rifampicin for recipient EC600). In addition, conjugal transfer of a well-studied SXT/R391 element ICE*Pmi*Jpn1 to *E. coli* EC600 was served as positive control in the conjugation experiments (Harada et al., 2010; Lei et al., 2016).

2.5. Nucleotide sequence accession number

The 100848-bp nucleotide sequence of ICE*Apl*Chn1 has been deposited in the GenBank database and assigned the accession no. KX196444.

3. Results and discussion

3.1. MDR profile of A. pleuropneumoniae app6

A. pleuropneumoniae strain app6 belongs to serovar 5, which is a prevalent serovar not only in China, but also the U.S., Canada, Brazil, Chile, Korea and Taiwan (Dubreuil et al., 2000). Susceptibility testing showed that strain app6 was resistant to florfenicol, chloramphenicol, tetracycline, amikacin, kanamycin, streptomycin, tilmicosin, erythromycin, clindamycin, lincomycin and sulfamethoxazole (Table 1), and susceptible to gentamicin (MIC, 8 mg/L), ampicillin (MIC, 0.125 mg/L), penicillin (MIC, 0.25 mg/L), ceftiofur (MIC, $\leq$0.032 mg/L), enrofloxacin (MIC, 0.125 mg/L) and tiamulin (MIC, 4 mg/L).

3.2. Structure of ICEAplChn1 and phylogenetic analysis

To investigate the genetic basis of the MDR phenotype of strain app6, whole genome sequencing was conducted and following assembly yielded 55 contigs of > 1000bp. By gap filling and comparison with the complete genome sequences of *A. pleuropneumoniae* strains deposited in the GenBank database, a novel ICE in the chromosome of *A. pleuropneumoniae* strain app6 was identified. This ICE element integrates into the 5′end of the *prfC* gene and possesses a conserved Int integrase, both of which are specific features of the SXT/R391 ICE