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Short Communication

Identification and characterization of new members of the SXT/R391 family of integrative and conjugative elements (ICEs) in *Proteus mirabilis*

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ABSTRACT

Integrative and conjugative elements (ICEs) are self-transmissible chromosomal mobile elements that play significant roles in the dissemination of antimicrobial resistance genes. Identification of the structures and functions of ICEs, particularly those in pathogens, improves understanding of the dissemination of antimicrobial resistance. This study identified new members of the sulfamethoxazole–trimethoprim (SXT)/R391 family of ICEs that could confer multi-drug resistance in the opportunistic pathogen *Proteus mirabilis*, characterized their genetic structures, and explored their evolutionary connection with other members of this family of ICEs. Three new members of the SXT/R391 family of ICEs were detected in six of 77 *P. mirabilis* strains isolated in China: ICE*Pmi*Chn2 (one strain), ICE*Pmi*Chn3 (one strain) and ICE*Pmi*Chn4 (three strains). All three new ICEs harbour antimicrobial resistance genes from diverse origins, suggesting their capability in acquiring foreign genes and serving as important carriers for antimicrobial resistance genes. Structural analysis showed that ICE*Pmi*Chn3 is a particularly interesting and unique ICE that has lost core genes involved in conjugation, and could not transfer to other cells via conjugation. This finding confirmed the key roles of these missing genes in conjugation. Further phylogenetic analysis suggested that ICEs in geographically close strains are also connected evolutionarily, and ICE*Pmi*Chn3 lost its conjugation cassette from a former mobile ICE. The identification and characterization of the three new members of the SXT/R391 family of ICEs in this work leads to suggestions of core ICE genes essential for conjugation, and extends understanding on the structures of ICEs, evolutionary relationships between ICEs, and the antimicrobial resistance mechanisms of *P. mirabilis*.

1. Introduction

Integrative and conjugative elements (ICEs) are a diverse group of mobile genetic elements that can transfer from cell to cell via conjugation, and lead to the dissemination of antimicrobial resistance [1]. The sulfamethoxazole–trimethoprim (SXT)/R391 family of ICEs, one of the largest and best-studied families of ICEs, share a conserved integrase that mediates site-specific integration into the *prfC* gene of the host chromosome, and are composed of 52 nearly identical core genes involved in integration/excision, conjugative transfer and regulation [2,3]. In addition, the SXT/R391 family of ICEs have five hotspots (HS1–HS5) and four variable regions (VRI–IV) in which variable genetic elements, including antimicrobial resistance gene arrays, are located. These genetic elements confer specific properties that regulate the adaptive function of their hosts. In particular, antimicrobial resistance (such as resistance to sulfamethoxazole, trimethoprim, streptomycin, chloramphenicol and kanamycin) is a common feature of these genetic elements [4,5].

Proteus mirabilis is an opportunistic nosocomial pathogen in humans and animals, and has been recognized to be a common cause of urinary tract infection and chronic otitis externa. It has been shown that antimicrobial-resistant *P. mirabilis* may cause a significant reduction in the number of antibiotics that can cure *P. mirabilis* infection [6–8]. To date, only five members of the SXT/R391 family of ICEs that could play a role in multi-drug resistance have been detected in *P. mirabilis*, although it is the most common family of ICEs discovered to date [9–13]. Therefore, more in-depth investigation of this important ICE family in *P. mirabilis* is warranted.

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2. Materials and methods

2.1. Bacterial isolation

Between June and September 2013, 77 antimicrobial-resistant *P. mirabilis* strains were isolated from a commercial broiler slaughter plant in Shandong Province, China, using the method described by Wu et al [14]. Antibiotics used for screening included amoxicillin–clavulanic acid (32/16 μg/mL), ciprofloxacin (4 mg/L), amikacin (32 mg/L), doxycycline (16 mg/L), sulbactam–cefoperazone (64/64 mg/L) and SXT (4/76 mg/L). The isolated strains were resistant to at least one of the antibiotics used.

2.2. Antimicrobial susceptibility assay

The antimicrobial sensitivity phenotypes of these ICE-harbouring strains were determined by the agar dilution method using Müller–Hinton agar plates following the guidelines of the Clinical Laboratory Standards Institute [15]. The following antimicrobial agents were used in addition to the antibiotics used for screening: ceftazidime (30 mg/L), ampicillin (100 mg/L), cefotaxime (30 mg/L), cefepime (30 mg/L), nalidixic acid (100 mg/L), ciprofloxacin (5 mg/L), streptomycin (10 mg/L), kanamycin (30 mg/L), tetracycline (30 mg/L), trimethoprim (30 mg/L), chloramphenicol (30 mg/L), erythrocin (15 mg/L), sulfisoxazole (250 mg/L), imipenem (10 mg/L) and meropenem (10 mg/L). The reference strain *Escherichia coli* ATCC 25922 was used as a negative control.

2.3. Screening and molecular identification of ICE-containing strains

The presence of ICEs from the SXT/R391 family in *P. mirabilis* strains was screened using polymerase chain reaction (PCR) with primers targeting the conserved *int* gene [12].

The clonal relationship of ICE-containing strains was determined by PCR–restriction fragment length polymorphism (PCR-RFLP) analysis of the O-antigen gene locus, as described previously [16].

2.4. Genome sequencing

The structures of ICEs were analysed by PCR-mapping DNA fragments inserted into the variable regions of ICEs. The complete sequences of ICEs were obtained by sequencing distinct ICE-containing *P. mirabilis* strains using the Illumina MiSeq (Illumina Inc., San Diego, CA, USA) sequencing platform with a 400-bp paired-end library, as well as SOAPdevono v2.04 and GapCloser v1.12 software to construct de-novo assemblies [17]. Gaps between scaffolds were closed by PCR followed by sequencing.

2.5. Conjugation of SXT/R391 family ICEs

Conjugation experiments were performed by the solid mating assay using isolated *P. mirabilis* strains as donor strains, and *E. coli* strain J53 as the recipient strain [18]. For ICE*Pmi*Chn2, ICE*Pmi*Chn3 and ICE*Pmi*Chn4, transconjugants were selected on MacConkey agar plates supplemented with sodium azide (300 mg/L) and chloramphenicol (30 mg/L), while sodium azide (300 mg/L) and ampicillin (100 mg/L) were used to screen the ICE*Pmi*Jpn1-harbouring transconjugants. The presence of members of the SXT/R391 family of ICEs in the transconjugants was detected by PCR targeting the ICE *int* gene, as well as antimicrobial resistance of transconjugants conferred by ICEs.

2.6. Bioinformatics

The complete nucleotide sequences of ICEs were analysed using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic tree construction was performed using the maximum likelihood method in MEGA 6.0 [19]. The GenBank accession numbers for ICE*Pmi*Chn2, ICE*Pmi*Chn3, ICE*Pmi*Chn4 and ICE*Pmi*Jpn1 are KY437726, KY437727, KY437728 and KY437729, respectively.

3. Results and discussion

Members of the SXT/R391 family of ICEs were detected in six *P. mirabilis* strains that were confirmed to be distinct clonal strains with PCR-RFLP analysis (Fig. S1). The DNA patterns inserted at each site of these ICEs were analysed, leading to the identification of four different members of the SXT/R391 family. The sequences of the four ICEs were further obtained by sequencing four distinct ICE-containing *P. mirabilis* strains: JN7, JN28, JN39 and JN49. Analysis of complete nucleotide sequences of ICEs confirmed the presence of four different members of the SXT/R391 family. The ICE in strain JN39 was identical to ICE*Pmi*Jpn1 reported previously [12], while the multidrug-resistant ICEs identified in JN7, JN28 and JN49 had new genetic structures. According to the nomenclature system proposed by Burrus et al [20], they were subsequently denominated ICE*Pmi*Chn2, ICE*Pmi*Chn3 and ICE*Pmi*Chn4, respectively (Fig. 1). The antibiotic resistance profiles of these six strains are listed in Table 1.

The acquisition of ICEs enables cells to display specific properties determined by the functional genes present in these ICEs. As shown in Fig. 1, ICE*Pmi*Chn2 and ICE*Pmi*Chn4 have identical genetic structures to ICE*Pmi*Chn1 at variable regions HS1, HS2 and HS4, and the gene organization at variable region III is similar in the three ICEs although the antimicrobial resistance genes in this region differ significantly [13]. Two additional genes, *chn4-1* and *chn4-2*, are present at variable region III in ICE*Pmi*Chn4, and these have not been detected previously in any member of the SXT/R391 family of ICEs. They encode a hypothetical protein and a β-lactamase, respectively,

Table 1
Members of the SXT/R391 family of integrative and conjugative elements (ICEs) in *P. mirabilis* isolates characterized in this study.

Strain	Antimicrobial resistance profile	Contained ICE	ICE size (bp)	Conjugative frequency
JN7	AMP, CTX, NAL, CIP, STR, KAN, TET, TMP, CHL, ERY, SFX, AMC, DOX, SXT	ICE*Pmi*Chn2	104,371	3.5×10^{-6}
JN14	NAL, CIP, STR, KAN, TET, TMP, CHL, ERY, SFX, IPM, DOX, SXT	ICE*Pmi*Chn4	90,566	6.2×10^{-7}
JN28	AMP, CTX, FEP, NAL, CIP, STR, KAN, TET, TMP, CHL, ERY, SFX, DOX, SXT	ICE*Pmi*Chn3	55,195	0
JN39	AMP, NAL, CIP, STR, KAN, TET, TMP, SFX, IPM, AMC, DOX, SXT	ICE*Pmi*Jpn1	91,091	2.6×10^{-6}
JN49	AMP, CTX, NAL, CIP, STR, KAN, TET, TMP, CHL, ERY, SFX, IPM, AMC, AMK, DOX, SXT	ICE*Pmi*Chn4	90,566	3.3×10^{-6}
JN77	AMP, CTX, FEP, NAL, STR, KAN, TET, TMP, CHL, ERY, SFX, IPM, AMC, AMK, DOX, SXT	ICE*Pmi*Chn4	90,566	1.2×10^{-6}

CAZ, ceftazidime; AMP, ampicillin; CTX, cefotaxime; FEP, cefepime; NAL, nalidixic acid; CIP, ciprofloxacin; STR, streptomycin; KAN, kanamycin; TET, tetracycline; TMP, trimethoprim; CHL, chloramphenicol; ERY, erythrocin; SFX, sulfisoxazole; IPM, imipenem; MEM, meropenem; AMC, amoxicillin–clavulanic acid; AMK, amikacin; DOX, doxycycline; SBT/CPZ, sulbactam–cefoperazone; SXT, sulfamethoxazole–trimethoprim.