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Chromosomally Encoded bla_{CMY-2} Located on a Novel SXT/R391-Related Integrating Conjugative Element in a *Proteus mirabilis* Clinical Isolate▽

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Integrating conjugative elements (ICEs) are mobile genetic elements that can transfer from the chromosome of a host to the chromosome of a new host through the process of excision, conjugation, and integration. Although SXT/R391-related ICEs, originally demonstrated in *Vibrio cholerae* O139 isolates, have become prevalent among *V. cholerae* isolates in Asia, the prevalence of the ICEs among Gram-negative bacteria other than *Vibrio* spp. remains unknown. In addition, SXT/R391-related ICEs carrying genes conferring resistance to extended-spectrum cephalosporins have never been described. Here we carried out a genetic analysis of a cefoxitin-resistant *Proteus mirabilis* clinical isolate, TUM4660, which revealed the presence of a novel SXT/R391-related ICE, ICE*Pmi*Jpn1. ICE*Pmi*Jpn1 had a core genetic structure showing high similarity to that of R391 and carried *xis* and *int* genes completely identical to those of R391, while an IS*10*-mediated composite transposon carrying bla_{CMY-2} was integrated into the ICE. A nucleotide sequence identical to the 3′ part of IS*Ecp1* was located upstream of the bla_{CMY-2} gene, and other genes observed around bla_{CMY-2} in earlier studies were also present. Furthermore, the nucleotide sequences of hot spot 2 and hot spot 4 in ICE*Pmi*Jpn1 showed high similarity to that of hot spot 2 in SXT^{MO10} and with a part of the nucleotide sequence found in *P. mirabilis* ATCC 29906, respectively. ICE*Pmi*Jpn1 was successfully transferred to *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella enterica* serovar Typhimurium, and *Citrobacter koseri* in conjugation experiments. These observations suggest that ICEs may contribute to the dissemination of antimicrobial resistance genes among clinically relevant *Enterobacteriaceae*, which warrants careful observation of the prevalence of ICEs, including SXT/R391-related ICEs.

Acquired AmpC enzymes have recently been distributed globally among nontyphoid *Salmonella* spp. (10, 15), *Escherichia coli* (20, 39), and other species of *Enterobacteriaceae* (16, 27). Most acquired *ampC* genes are highly expressed, presumably because of the possession of promoters of greater strength than those for chromosomally encoded enzymes and because of the multiple copy numbers of the relevant plasmid, rendering the host organisms resistant to a variety of β-lactams, including oxyimino-cephalosporins and cephamycins (23). CMY-2 and its derivatives, presumably derived from the chromosomally encoded AmpC protein of *Citrobacter freundii*, appear to be the most common acquired AmpC enzymes worldwide (23). IS*Ecp1* has frequently been found upstream of bla_{CMY-2}-like genes in full-length or partially inverted form or with insertions of other transposons, such as IS*5*, IS*10*, or IS*1294*, and is assumed to be involved in mobilization and expression of bla_{CMY-2}-like genes (25, 42).

Proteus mirabilis is a common causative organism of urinary tract infections in patients with indwelling urinary catheters or with anatomic or functional abnormalities of the urinary tract (22). Although *P. mirabilis* originally lacks a chromosomal β-lactamase gene and is highly susceptible to β-lactams (30), the production of β-lactamases, including AmpC enzymes and extended-spectrum β-lactamases (ESBLs), by *P. mirabilis* clinical isolates, through acquisition of resistance genes by horizontal gene transfer, has been reported in recent years (16, 36). Chromosomal acquisition of bla_{CMY} has also been demonstrated in some studies; however, the mechanism by which the resistance genes were acquired remains unknown (6, 14, 28).

Integrating conjugative elements (ICEs) are self-transmissible genetic elements with the abilities to excise themselves from the chromosome of their host cell to form a circular intermediate, to transfer to another cell by conjugation, and to integrate themselves into the chromosome of the new host cell (7). SXT^{MO10} is an ICE carrying genes mediating resistance to chloramphenicol, sulfamethoxazole, trimethoprim, and streptomycin and was originally discovered in *Vibrio cholerae* O139, a newly recognized epidemic strain which emerged in India in the early 1990s (43). While SXT-related ICEs were rarely found in *V. cholerae* O1 isolates before the emergence of *V. cholerae* O139, most *V. cholerae* O1 isolates in Asia, together with O139 isolates, now seem to carry SXT-related ICEs (2, 19, 21). Furthermore, SXT-related ICEs have been found in *V. cholerae* isolates outside Asia (8, 13), in *Vibrio* species other than *V. cholerae* (1, 40), and in other gammaproteobacteria (35, 38).

R391 is an ICE discovered in a *Providencia rettgeri* clinical isolate in South Africa (12). Although R391 was once described as a conjugative plasmid of the "IncJ" incompatibility group, recent genetic analysis revealed that R391 is an 89-kb ICE which has high genetic and functional similarity to

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TABLE 1. Bacterial strains used in this study

Strain	Characteristics	Reference or source
P. mirabilis TUM4660	Clinical isolate showing reduced susceptibility to extended-spectrum cephalosporins	This study
E. coli ML4909	F$^-$ *galK2 galT22 hsdR metB1 relA supE44*; rifampin resistant	M. Inoue
E. coli ML4901	F$^-$ *galK2 galT22 hsdR lacY1 metB1 relA supE44*; nalidixic acid resistant	M. Inoue
K. pneumoniae TUM5459	Rifampin-resistant mutant of *K. pneumoniae* ATCC 13883	This study
S. Typhimurium TUM5460	Rifampin-resistant mutant of *S.* Typhimurium ATCC 14028	This study
C. koseri TUM5461	Rifampin-resistant mutant of *C. koseri* ATCC 27028	This study
E. coli TUM4670	ICE*Pmi*Jpn1 transconjugant of ML4909	This study
E. coli TUM4672	ICE*Pmi*Jpn1 transconjugant of ML4901	This study

SXT^{MO10} (4, 5). SXT/R391-related ICEs have a highly conserved core set of genes, including *int*, *xis*, and *tra* genes (7). Int and Xis mediate integration and excision of the element. Tra proteins of SXT^{MO10} have significant homology with those encoded by several conjugative plasmids, and excised circular intermediates of SXT/R391-related ICEs appear to transfer to other cells by a mechanism which is similar to that used by conjugative plasmids. It has been documented that SXT/R391-related ICEs are integrated specifically into the 5′ end of the *prfC* gene, encoding peptide chain release factor 3 (3). In addition to conserved core genes, each SXT/R391-related ICE has specific genes conferring specific properties of the ICE, such as antimicrobial resistance. Interestingly, comparison of the nucleotide sequences of four fully sequenced SXT/R391-related ICEs revealed that most of such additional genes specific to each ICE are located at several specific sites, namely, four hot spots and several sites between *attL* and *traI* (7, 35, 38).

In this study, we describe a *P. mirabilis* clinical isolate carrying bla_{CMY-2} on a novel SXT/R391-related ICE, designated ICE*Pmi*Jpn1, and analyze the genetic structure of the ICE.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table 1. *P. mirabilis* TUM4660 was isolated in 2006 from a wound swab culture from a patient with soft tissue infection hospitalized in a tertiary care hospital in Japan. *E. coli* ML4909 and *E. coli* ML4901, which were used as recipients in conjugation experiments, were provided by Matsuhisa Inoue of Kitasato University. *Klebsiella pneumoniae* ATCC 13883, *Salmonella enterica* serovar Typhimurium ATCC 14028, and *Citrobacter koseri* ATCC 27028 were purchased from the American Type Culture Collection, and their *in vitro*-obtained rifampin-resistant mutants, TUM5459, TUM5460, and TUM5461, respectively, were used as recipients in conjugation experiments.

Antibacterial agents. The following antimicrobials were used for antibiotic susceptibility testing and/or selection of transconjugants. Amoxicillin, cefotaxime, ceftazidime, cefoxitin, gentamicin, trimethoprim, nalidixic acid, rifampin, and ciprofloxacin were purchased from Sigma Chemical Co. (St. Louis, MO). Cefepime was from Bristol-Myers Squibb (Tokyo, Japan). Imipenem was from Banyu Pharmaceutical Co., Ltd. (Tokyo, Japan). Clavulanic acid was from GlaxoSmithKline K.K. (Tokyo, Japan). Tazobactam and piperacillin were from Taisho Toyama Pharmaceutical Co., Ltd. (Tokyo, Japan).

Susceptibility testing. MICs were determined by broth dilution methods according to the guidelines of the Clinical and Laboratory Standards Institute (11). MICs of several β-lactams were determined alone or in combination, with a fixed concentration of clavulanic acid (2 μg/ml) or tazobactam (4 μg/ml). Disk potentiation tests, using Kirby-Bauer (KB) disks (Eiken Chemical Co., Ltd., Tokyo, Japan) containing 30 μg of ceftazidime alone or with 300 μg of 3-aminophenylboronic acid (APB) (Sigma Chemical Co.), were performed to identify the production of AmpC β-lactamase (44).

DNA preparation and identification of β-lactamase gene. Isolation of genomic DNA was carried out with a Sepagene kit (Sanko Junyaku, Tokyo, Japan). The *ampC* gene was amplified by PCR with primers *amp*C1 and *amp*C2 (Table 2) (26). The PCR product was purified with a QIAquick PCR purification kit (Qiagen, Hilden, Germany) and subsequently sequenced with an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA).

Conjugation experiments. Conjugation experiments were performed by filter mating methods. Transconjugants were selected on bromothymol blue-lactose agar supplemented with cefotaxime (4 μg/ml) and either rifampin (50 μg/ml) or nalidixic acid (100 μg/ml).

PCR for genetic structures of SXT/R391-related ICEs. The involvement of SXT/R391-related ICEs in conjugative transfer of bla_{CMY-2} from *P. mirabilis* TUM4660 to *E. coli* ML4909 was analyzed by molecular methods as described by McGrath et al. (32, 33). PCR amplification of *attL* and *attR* junctions in *E. coli* was attempted with primer pair LE1 and LE4 and primer pair RE1 and RE4, respectively (Table 2). Primer pair PMLE1 and LE4 and primer pair PMRE1 and RE4 were used to amplify *attL* and *attR* junctions in *P. mirabilis*, respectively (Table 2). The presence of an excised circular form of SXT/R391-related ICE was examined by PCR with primers LE4 and RE4. The *int* gene of SXT/R391-related ICEs was amplified with the primers IntFor1 and IntRev1 (Table 2).

Plasmid analysis and I-CeuI experiments. Plasmid DNAs were extracted by alkaline lysis according to the method described by Kado and Liu (24). After Southern transfer to a Hybond-N$^+$ membrane (GE Healthcare, Little Chalfont, United Kingdom), the extracted plasmid of TUM4660 was hybridized with a bla_{CMY-2}-specific probe generated by PCR with primers *amp*C1 and *amp*C2 (Table 2). Labeling, hybridization, and immunological detection were performed with a DIG High Prime DNA labeling and detection starter kit I (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. A possible chromosomal location of the *ampC* gene was analyzed with the I-CeuI technique (29, 34). After digestion of whole-cell DNA with I-CeuI (New England Biolabs, Hertfordshire, United Kingdom), the resultant fragments were separated with a CHEF-Mapper apparatus (Bio-Rad, Hercules, CA) at 14°C, 6 V/cm, and a 120° switch angle, with a nonlinear switch time ramp of 5.3 to 49.9 s for 19.7 h. The sizes of the fragments were determined by comparison with a yeast chromosome pulsed-field gel electrophoresis marker (New England Biolabs). After Southern transfer to a Hybond-N$^+$ membrane, the fragments were hybridized with three different PCR-generated probes: the probe for *ampC*, described above; a probe for the 23S rRNA gene, generated with primers 23S-For and 23S-Rev (Table 2); and a probe for *int*, generated with primers IntFor1 and IntRev1.

Genetic characterization of ICE*Pmi*Jpn1. The genetic structure of the R391/SXT-related ICE in *P. mirabilis* TUM4660, ICE*Pmi*Jpn1, was analyzed by PCR amplification of variable regions of the ICE with primers designed according to the nucleotide sequences conserved among R391/SXT-related ICEs (Table 2). Extracted genomic DNAs from *P. mirabilis* TUM4660 and *E. coli* TUM4670 were used as DNA templates to confirm that they produced the same amplicon by PCR. PCR products were purified and sequenced. Nucleotide sequences were analyzed with BLAST at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

Nucleotide sequence accession numbers. The nucleotide sequences described in this article have been deposited in GenBank under accession numbers AB525688 (*attL* to *traI*), AB525225 (hot spot 1), AB525226 (hot spot 2), AB525227 (hot spot 4), AB525228 (hot spot 3), and AB525230 (*attR*).

RESULTS

Properties of *P. mirabilis* TUM4660 isolate. *P. mirabilis* TUM4660 exhibited resistance to cefoxitin, ceftazidime, and cefotaxime. Clavulanic acid showed no synergistic effect on the susceptibilities to ceftazidime and cefotaxime (Table 3). The addition of 300 μg of APB to KB disks containing ceftazidime

TABLE 2. Primers used in this study

Primer	Nucleotide sequence (5′ to 3′)	Region amplified	Reference
ampC1	ATGATGAAAAAATCGTTATGC	*ampC*	26
ampC2	TTGCAGCTTTTCAAGAATGCGC	*ampC*	26
23S-For	AATGATGGCCAGGCTGTCTCC	23S rRNA	This study
23S-Rev	CCGCCGTCGATATGAACTCTTG	23S rRNA	This study
LE4	GTACACACTTTCCGAGGTTACG	*attL*	8
LE1	GTTTCTTCGTTGCACGAACTGG	*attL*	8
PMLE1	ACAACGACAACAGAGCATTGG	*attL*	This study
RE4	CCGCAATACCCTGCAATACCGA	*attR*	8
RE1	CGGTCTGAATGGCCTGTCCGAA	*attR*	8
PMRE1	TGCACGTTGGATAGCTTGTCCG	*attR*	This study
intFor1	AAACTAGGGCTGGGCTTATAACATGGCG	*int*	32
intRev1	AAAGATGGCAGCTTGCCGCAACCTC	*int*	32
orf14-Rev2	CTGGTAGCAATGGGAGCATC	*int-orf14*	This study
orf14-For1	TTCGTCTCTGCCCGCATCATTG	*orf14-bla*$_{CMY-2}$	This study
CMY-Rev	ACGTCATCGGGGATCTGCAGCG	*orf14-bla*$_{CMY-2}$	This study
CMY-For	TTATTTCACCTGGGTAAAGCC	*bla*$_{CMY-2}$*-orf14*	This study
orf14-Rev1	TTTCTGGCCTTGATAGAAGCTC	*bla*$_{CMY-2}$*-orf14*	This study
orf14-For2	GCTAGGCGTGTTCGAGAGAATG	*orf14-traI*	This study
traI-Rev	GGCAATTAGCTCGTCTTGTGTG	*orf14-traI*	This study
HS1F	GGCTATTCCACCGGTGGTG	Hot spot 1	8
HS1R	TGCCGATCACTAGCCCCAAC	*orf36-traL*	8
HotS2F	TTCCAGCAATCAGCGCCG	Hot spot 2	8
HotS2R	CAGTTGTCCTATGTGGACTCGG	*traA-orf55*	8
HotS4F	ATTGAACGGCTAAACGGAATGG	Hot spot 4	This study
HotS4R	GCACGAAAATCAGCCCAAGC	*traN-orf65*	This study
HotS3F2	CCTAAGCATCCTTGGAAGGCT	Hot spot 3	8
TraF2R	TGGGATGGTCACCCATAGGA	*orf79-traF*	8

resulted in enlargement of the inhibitory zone diameter by >5 mm compared with that on KB disks alone, so the production of a class C β-lactamase by TUM4660 was suggested. Amplification of genomic DNA of TUM4660 with primers *amp*C1 and *amp*C2 was successful, and sequencing of the PCR product confirmed the presence of bla_{CMY-2} in TUM4660.

Transfer of β-lactam resistance. An *E. coli* ML4909 transconjugant of *P. mirabilis* TUM4660, TUM4670, was obtained at a frequency of 10^{-9} per donor and showed a broad-spectrum β-lactam resistance phenotype similar to that of TUM4660 (Table 3). A conjugation experiment with *E. coli* TUM4670 and *E. coli* ML4901 was successful at a higher frequency (10^{-5} per donor), and *E. coli* TUM4672 was obtained as a transconjugant. Conjugative transfer of bla_{CMY-2} was also successful from *E. coli* TUM4672 to *K. pneumoniae* TUM5459, *S.* Typhimurium TUM5460, and *C. koseri* TUM5461.

TABLE 3. Results of antibiotic susceptibility testing

Antimicrobial agent[a]	MIC (μg/ml)				
	P. mirabilis TUM4660	*E. coli* TUM4670	*E. coli* TUM4672	*E. coli* ML4909	*E. coli* ML4901
Amoxicillin	>128	>128	>128	2	4
Amoxicillin-CLA	>128	>128	>128	2	4
Piperacillin	>128	>128	>128	2	2
Piperacillin-TZB	16	>128	>128	2	2
Cefotaxime	8	16	32	≤0.06	≤0.06
Cefotaxime-CLA	16	32	64	≤0.06	≤0.06
Ceftazidime	16	128	128	0.25	0.25
Ceftazidime-CLA	16	32	64	0.12	≤0.06
Cefepime	2	1	2	≤0.06	≤0.06
Cefoxitin	128	128	>128	4	2
Imipenem	8	0.25	0.25	0.12	0.12
Gentamicin	1	1	0.5	2	2
Trimethoprim	4	≤0.06	≤0.06	0.12	≤0.06
Ciprofloxacin	0.12	≤0.06	≤0.06	≤0.06	≤0.06

[a] CLA, clavulanic acid at a fixed concentration of 2 μg/ml; TZB, tazobactam at a fixed concentration of 4 μg/ml.

Determination of the location of the bla_{CMY-2} gene in *P. mirabilis* TUM4660. Although TUM4660 carried a 7-kb plasmid, no plasmids were extracted from *E. coli* TUM4670 by the same procedure. Furthermore, the plasmid of TUM4660 did not hybridize with a bla_{CMY-2}-specific probe (data not shown). Hybridization experiments after I-CeuI digestion of genomic DNA demonstrated that two copies of bla_{CMY-2} were located on the chromosome of TUM4660 and that bla_{CMY-2} was also located on the chromosome of TUM4670 (Fig. 1). Therefore, it was suggested that transfer of bla_{CMY-2} from the chromosome of TUM4660 to the chromosome of *E. coli* ML4909 occurred in the conjugation experiment.

Involvement of R391/SXT-related ICE in the conjugative transfer of bla_{CMY-2}. PCR amplification of the genomic DNA of TUM4670 with primer pair LE1/LE4 or RE1/RE4 yielded a 347-bp amplicon or a 454-bp amplicon, respectively, while the results for PCR amplification of the genomic DNA of ML4909 were negative. These results suggested that an SXT/R391-related ICE was integrated into the 5′ end of the *prfC* gene in TUM4670 during the conjugation experiment. Integration of an SXT/R391-related ICE into the 5′ end of the *prfC* gene in TUM4660 was also suggested by the positive results of PCRs with primers PMLE1 and LE4 and primers PMRE1 and RE4. PCR with *int*-specific primers and sequencing of the amplicon demonstrated the presence of an *int* gene that was 100% identical to that of R391 in TUM4660. PCR with primers LE4 and

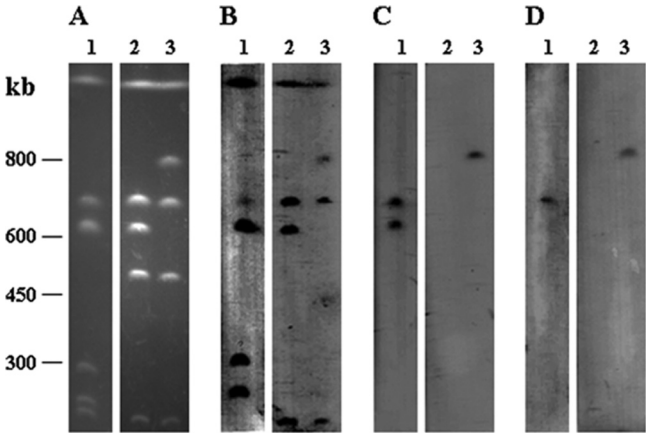

FIG. 1. Localization of bla_{CMY-2} and *int* in *P. mirabilis* TUM4660 and its transconjugant. (A) Whole genomic DNAs of *P. mirabilis* TUM4660 (lane 1), *E. coli* ML4909 (lane 2), and *E. coli* TUM4670 (lane 3) were digested with I-CeuI, and the restricted fragments were subjected to pulsed-field gel electrophoresis. DNA fragments were transferred to a nylon membrane and hybridized with probes specific to the 23S rRNA gene (B), bla_{CMY-2} (C), and *int* (D).

RE4 yielded a 542-bp product when the genomic DNA of TUM4660 or TUM4670 was used as template DNA, whereas the result was negative for ML4909. This suggested the presence of the excised circular form of an ICE in TUM4660 and TUM4670.

The results of hybridization studies with an *int*-specific probe supported the hypothesis of the conjugative transfer of bla_{CMY2} involving an SXT/R391-related ICE (Fig. 1). The SXT/R391-related ICE responsible for the transfer of bla_{CMY-2} was designated ICE*Pmi*Jpn1 according to the nomenclature proposed by Burrus et al. (7), indicating the first SXT/R391-related ICE of *P. mirabilis* found in Japan. An additional copy of bla_{CMY-2} unrelated to the SXT/R391-related ICE was also located on the chromosome of TUM4660.

Genetic characterization of ICE*Pmi*Jpn1. The nucleotide sequences of the *attL-traI* region and the four hot spots of ICE*Pmi*Jpn1 were determined (Fig. 2). Although the nucleotide sequence of ICE*Pmi*Jpn1 from *attL* to *traI* showed high similarity to that of R391, several open reading frames (ORFs) which did not exist in R391 were found. ICE*Pmi*Jpn1 carried an IS*10*-mediated composite transposon inserted into *orf14* of R391. This transposon was composed of two copies of IS*10* accompanied by target site 9-bp duplications on both ends and several ORFs, including that encoding the CMY-2 enzyme. A nucleotide sequence identical to the 3′ part of IS*Ecp1* was located 117 bp upstream from bla_{CMY-2}. On the other hand, a composite transposon in R391 with a kanamycin resistance gene surrounded by three copies of IS*15* did not exist in ICE*Pmi*Jpn1. Although the nucleotide sequences of hot spot 1 and hot spot 4 showed high similarities with those in R391 (96% and 99%, respectively), the nucleotide sequence of

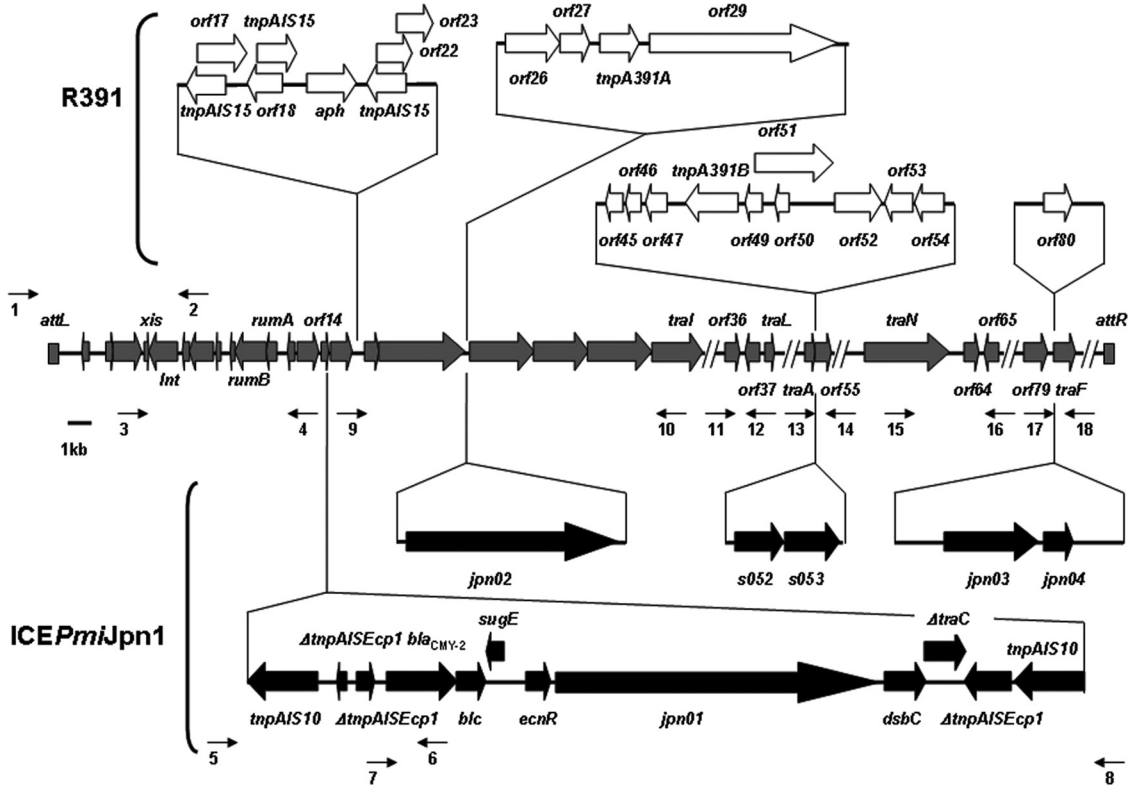

FIG. 2. Schematic representation of genes conserved between ICE*Pmi*Jpn1 and R391 and genes specific to ICE*Pmi*Jpn1 and R391. The gray arrows represent genes conserved between ICE*Pmi*Jpn1 and R391. The black and white arrows represent genes specific to ICE*Pmi*Jpn1 and R391 (not to scale), respectively. Thin black arrows represent primers used for PCR amplification of variable regions. Primers: 1, PMLE1; 2, intFor1; 3, intRev1; 4, orf14-Rev2; 5, orf14-For1; 6, CMY-Rev; 7, CMY-For; 8, orf14-Rev1; 9, orf14-For2; 10, traI-Rev; 11, HS1F; 12, HS1R; 13, HotS2F; 14, HotS2R; 15, HotS4F; 16, HotS4R; 17, HotS3F2; 18, TraF2R.

hot spot 2 was almost identical (98%) to that of SXT^{MO10}. Hot spot 3 possessed 2 ORFs, Jpn03 and Jpn04, which have high similarities with ORFs encoding the 5-methylcytosine-specific restriction enzyme B and a conserved hypothetical protein of *P. mirabilis* ATCC 29906 (98% and 97%, respectively).

DISCUSSION

In this study, we characterized a novel SXT/R391-related ICE, ICE*Pmi*Jpn1, carrying bla_{CMY-2} on the chromosome of the *P. mirabilis* clinical isolate TUM4660. To the best of our knowledge, ICE*Pmi*Jpn1 is the first SXT/R391-related ICE carrying a gene conferring resistance to extended-spectrum cephalosporins.

ICE*Pmi*Jpn1 had *int* and *xis* genes completely identical to those of R391, and the nucleotide sequence between *attL* and *traI* also showed high similarity to that of R391 (Fig. 2). Nevertheless, several major differences, which characterize the properties of each ICE, were found between the nucleotide sequences of ICE*Pmi*Jpn1 and R391. ICE*Pmi*Jpn1 lacked the IS*15*-mediated composite transposon carrying a kanamycin resistance gene, whereas it possessed the IS*10*-mediated composite transposon of 14.2 kb carrying bla_{CMY-2}. The nucleotide sequence between the two copies of IS*10* showed >99% similarity to the nucleotide sequence in the 13-kb type I structure frequently found on bla_{CMY-2}-carrying plasmids in *E. coli* and *Salmonella enterica* isolates from humans and animals in the United States (25, 42). This structure contained a 2,823-bp region which appeared to be involved in IS*Ecp1*-mediated mobilization of the bla_{CMY-2}-like gene from the *C. freundii* chromosome, including IS*Ecp1*, 117 bp upstream from bla_{CMY-2}, and *blc*, *sugE*, and *ecnR*, which are downstream of bla_{CMY-2} (42). Verdet et al. recently reported that a clinical isolate of *P. mirabilis* RPCMY isolated in France carried a plasmid containing the 13-kb type I structure flanked by a copy of IS*10* at its 5′ end (42). We also identified a *P. mirabilis* isolate carrying a plasmid with the same genetic structure from retail chicken sampled in Japan (GenBank accession no. AB525078) (unpublished data). These findings suggest that a part of the 13-kb type I structure, once constructed on a relevant plasmid involving the acquisition of a part of the *C. freundii* chromosome, was employed in the prototypical ICE of ICE*Pmi*Jpn1 through IS*10*-mediated transposition.

In ICE*Pmi*Jpn1, the nucleotide sequences of hot spot 1 and hot spot 4 showed high similarities to those in R391, while the nucleotide sequence of hot spot 2 showed high similarity to that of SXT^{MO10}. Hot spot 3 carried nucleotide sequences unrelated to those of R391 or SXT^{MO10}. A similar situation was demonstrated in ICE*Vch*Lao1, which carried an R391-like insertion in hot spot 1, an SXT^{MO10}-like insertion in hot spot 2, and the original insertion in hot spot 3 (7, 41). Despite using an identical integration site, namely, the 5′ end of *prfC*, on the chromosome of the host cell, R391 and SXT^{MO10} can coexist in a cell and form a tandem array under experimental conditions (18). Formation of a hybrid ICE possessing R391-like nucleotide sequences in one part and SXT^{MO10}-like nucleotide sequences in another part was observed in transconjugants derived from donor cells containing tandem arrays of R391 and SXT^{MO10} (9). This phenomenon may explain the mechanism that generated the mosaic structure observed in ICE*Pmi*Jpn1 and ICE*Vch*Lao1 and may contribute to the diversity of SXT/R391-related ICEs.

Although the presence of several IncJ elements was demonstrated for several isolates of *Proteus* spp. in the 1970s (17, 31), the presence of novel ICEs in *Proteus* spp. was not reported until recently. Pearson et al. analyzed the complete genome sequence of *P. mirabilis* HI4320, a representative strain cultured from the urine of a patient with a long-term indwelling urinary catheter, and identified the presence of an ICE similar to R391 (37). Although the unintended detection of an SXT/R391-related ICE in the representative strain of *P. mirabilis* might suggest that the presence of SXT/R391-related ICEs in *P. mirabilis* clinical isolates is common, the prevalence of SXT/R391-related ICEs among *P. mirabilis* isolates has remained undetermined so far. The presence of an ICE could be overlooked if the ICE does not confer specific phenotypic properties, such as antimicrobial resistance, on the host. Even if it provides antimicrobial resistance to the host, one may wrongly assume that the antimicrobial resistance gene is located on a conjugative plasmid, without an active search for the involvement of an ICE, because of the ability to transfer the gene conferring antimicrobial resistance through conjugation.

Host range is one of the critical factors in determining the impact of a mobile genetic element conferring antimicrobial resistance. McGrath et al. identified *prfC* homologues in many different genera and found by an *in silico* search that a 12-bp section of the 17-bp integration site of R391 is conserved among 15 members of the *Gammaproteobacteria*. They demonstrated that up to a 23.5% mismatch between *attP* and *attB* was allowed in the integration process of R391, and they succeeded in conjugative transfer of R391 into *S.* Typhimurium, *Enterobacter cloacae*, and *Serratia marcescens* (33). We showed in this study that ICE*Pmi*Jpn1 can be transferred to several species of the *Enterobacteriaceae*. Given this broad potential host range and the selective advantage in the presence of antibiotics, ICE*Pmi*Jpn1 or other SXT/R391-related ICEs conferring antimicrobial resistance could play an important role in spreading antimicrobial resistance genes among clinically relevant *Enterobacteriaceae* in the near future, as already observed among *Vibrio* species globally (1, 2, 8, 13, 19, 21, 40).

In conclusion, this study demonstrated the involvement of a novel SXT/R391-related ICE, ICE*Pmi*Jpn1, in the acquisition of bla_{CMY-2} by a *P. mirabilis* clinical isolate. Although this is the first report of an ICE conferring resistance to extended-spectrum cephalosporins, extensive surveillance for the presence of ICEs has never been attempted. The mobile nature of ICEs warrants further studies to clarify the impact of ICEs on the spread of antimicrobial resistance genes in the *Enterobacteriaceae*.

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REFERENCES

1. **Ahmed, A., S. Shinoda, and T. Shimamoto.** 2005. A variant type of *Vibrio cholerae* SXT element in a multidrug-resistant strain of *Vibrio fluvialis*. FEMS Microbiol. Lett. **242:**241–247.
2. **Amita, S. Chowdhury, M. Thungapathra, T. Ramamurthy, G. Nair, and A. Ghosh.** 2003. Class I integrons and SXT elements in El Tor strains isolated before and after 1992 *Vibrio cholerae* O139 outbreak, Calcutta, India. Emerg. Infect. Dis. **9:**500–502.
3. **Beaber, J., V. Burrus, B. Hochhut, and M. Waldor.** 2002. Comparison of SXT and R391, two conjugative integrating elements: definition of a genetic backbone for the mobilization of resistance determinants. Cell. Mol. Life Sci. **59:**2065–2070.
4. **Beaber, J., B. Hochhut, and M. Waldor.** 2002. Genomic and functional analyses of SXT, an integrating antibiotic resistance gene transfer element derived from *Vibrio cholerae*. J. Bacteriol. **184:**4259–4269.
5. **Böltner, D., C. MacMahon, J. Pembroke, P. Strike, and A. Osborn.** 2002. R391: a conjugative integrating mosaic comprised of phage, plasmid, and transposon elements. J. Bacteriol. **184:**5158–5169.
6. **Bret, L., C. Chanal-Claris, D. Sirot, E. Chaibi, R. Labia, and J. Sirot.** 1998. Chromosomally encoded AmpC-type β-lactamase in a clinical isolate of *Proteus mirabilis*. Antimicrob. Agents Chemother. **42:**1110–1114.
7. **Burrus, V., J. Marrero, and M. Waldor.** 2006. The current ICE age: biology and evolution of SXT-related integrating conjugative elements. Plasmid **55:** 173–183.
8. **Burrus, V., R. Quezada-Calvillo, J. Marrero, and M. Waldor.** 2006. SXT-related integrating conjugative element in New World *Vibrio cholerae*. Appl. Environ. Microbiol. **72:**3054–3057.
9. **Burrus, V., and M. Waldor.** 2004. Formation of SXT tandem arrays and SXT-R391 hybrids. J. Bacteriol. **186:**2636–2645.
10. **Carattoli, A., F. Tosini, W. Giles, M. Rupp, S. Hinrichs, F. Angulo, T. Barrett, and P. Fey.** 2002. Characterization of plasmids carrying CMY-2 from expanded-spectrum cephalosporin-resistant *Salmonella* strains isolated in the United States between 1996 and 1998. Antimicrob. Agents Chemother. **46:**1269–1272.
11. **Clinical and Laboratory Standards Institute.** 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 7th ed. Document M7-A7. CLSI, Wayne, PA.
12. **Coetzee, J., N. Datta, and R. Hedges.** 1972. R factors from *Proteus rettgeri*. J. Gen. Microbiol. **72:**543–552.
13. **Dalsgaard, A., A. Forslund, D. Sandvang, L. Arntzen, and K. Keddy.** 2001. *Vibrio cholerae* O1 outbreak isolates in Mozambique and South Africa in 1998 are multiple-drug resistant, contain the SXT element and the *aadA2* gene located on class 1 integrons. J. Antimicrob. Chemother. **48:**827–838.
14. **D'Andrea, M., E. Nucleo, F. Luzzaro, T. Giani, R. Migliavacca, F. Vailati, V. Kroumova, L. Pagani, and G. Rossolini.** 2006. CMY-16, a novel acquired AmpC-type β-lactamase of the CMY/LAT lineage in multifocal monophyletic isolates of *Proteus mirabilis* from northern Italy. Antimicrob. Agents Chemother. **50:**618–624.
15. **Egorova, S., M. Timinouni, M. Demartin, S. Granier, J. Whichard, V. Sangal, L. Fabre, A. Delauné, M. Pardos, Y. Millemann, E. Espié, M. Achtman, P. Grimont, and F. Weill.** 2008. Ceftriaxone-resistant *Salmonella enterica* serotype Newport, France. Emerg. Infect. Dis. **14:**954–957.
16. **Empel, J., A. Baraniak, E. Literacka, A. Mrówka, J. Fiett, E. Sadowy, W. Hryniewicz, and M. Gniadkowski.** 2008. Molecular survey of β-lactamases conferring resistance to newer β-lactams in *Enterobacteriaceae* isolates from Polish hospitals. Antimicrob. Agents Chemother. **52:**2449–2454.
17. **Hedges, R.** 1975. R factors from *Proteus mirabilis* and *P. vulgaris*. J. Gen. Microbiol. **87:**301–311.
18. **Hochhut, B., J. Beaber, R. Woodgate, and M. Waldor.** 2001. Formation of chromosomal tandem arrays of the SXT element and R391, two conjugative chromosomally integrating elements that share an attachment site. J. Bacteriol. **183:**1124–1132.
19. **Hochhut, B., Y. Lotfi, D. Mazel, S. Faruque, R. Woodgate, and M. Waldor.** 2001. Molecular analysis of antibiotic resistance gene clusters in *Vibrio cholerae* O139 and O1 SXT constins. Antimicrob. Agents Chemother. **45:**2991–3000.
20. **Hopkins, K., M. Batchelor, E. Liebana, A. Deheer-Graham, and E. Threlfall.** 2006. Characterisation of CTX-M and AmpC genes in human isolates of *Escherichia coli* identified between 1995 and 2003 in England and Wales. Int. J. Antimicrob. Agents **28:**180–192.
21. **Iwanaga, M., C. Toma, T. Miyazato, S. Insisiengmay, N. Nakasone, and M. Ehara.** 2004. Antibiotic resistance conferred by a class I integron and SXT constin in *Vibrio cholerae* O1 strains isolated in Laos. Antimicrob. Agents Chemother. **48:**2364–2369.
22. **Jacobsen, S., D. Stickler, H. Mobley, and M. Shirtliff.** 2008. Complicated catheter-associated urinary tract infections due to *Escherichia coli* and *Proteus mirabilis*. Clin. Microbiol. Rev. **21:**26–59.
23. **Jacoby, G.** 2009. AmpC β-lactamases. Clin. Microbiol. Rev. **22:**161–182.
24. **Kado, C., and S. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145:**1365–1373.
25. **Kang, M., T. Besser, and D. Call.** 2006. Variability in the region downstream of the bla_{CMY-2} β-lactamase gene in *Escherichia coli* and *Salmonella enterica* plasmids. Antimicrob. Agents Chemother. **50:**1590–1593.
26. **Koeck, J. L., G. Arlet, A. Philippon, S. Basmaciogullari, H. V. Thien, Y. Buisson, and J. D. Cavallo.** 1997. A plasmid-mediated CMY-2 β-lactamase from an Algerian clinical isolate of *Salmonella senftenberg*. FEMS Microbiol. Lett. **152:**255–260.
27. **Li, Y., Q. Li, Y. Du, X. Jiang, J. Tang, J. Wang, G. Li, and Y. Jiang.** 2008. Prevalence of plasmid-mediated AmpC β-lactamases in a Chinese university hospital from 2003 to 2005: first report of CMY-2-type AmpC β-lactamase resistance in China. J. Clin. Microbiol. **46:**1317–1321.
28. **Literacka, E., J. Empel, A. Baraniak, E. Sadowy, W. Hryniewicz, and M. Gniadkowski.** 2004. Four variants of the *Citrobacter freundii* AmpC-type cephalosporinases, including novel enzymes CMY-14 and CMY-15, in a *Proteus mirabilis* clone widespread in Poland. Antimicrob. Agents Chemother. **48:**4136–4143.
29. **Liu, S., A. Hessel, and K. Sanderson.** 1993. Genomic mapping with I-Ceu I, an intron-encoded endonuclease specific for genes for ribosomal RNA, in *Salmonella spp.*, *Escherichia coli*, and other bacteria. Proc. Natl. Acad. Sci. U. S. A. **90:**6874–6878.
30. **Livermore, D.** 1995. β-Lactamases in laboratory and clinical resistance. Clin. Microbiol. Rev. **8:**557–584.
31. **Matthew, M., R. Hedges, and J. Smith.** 1979. Types of β-lactamase determined by plasmids in gram-negative bacteria. J. Bacteriol. **138:**657–662.
32. **McGrath, B., J. O'Halloran, A. Piterina, and J. Pembroke.** 2006. Molecular tools to detect the IncJ elements: a family of integrating, antibiotic resistant mobile genetic elements. J. Microbiol. Methods **66:**32–42.
33. **McGrath, B., and J. Pembroke.** 2004. Detailed analysis of the insertion site of the mobile elements R997, pMERPH, R392, R705 and R391 in *E. coli* K12. FEMS Microbiol. Lett. **237:**19–26.
34. **Naas, T., D. Aubert, A. Ozcan, and P. Nordmann.** 2007. Chromosome-encoded narrow-spectrum Ambler class A β-lactamase GIL-1 from *Citrobacter gillenii*. Antimicrob. Agents Chemother. **51:**1365–1372.
35. **Osorio, C., J. Marrero, R. Wozniak, M. Lemos, V. Burrus, and M. Waldor.** 2008. Genomic and functional analysis of ICE*Pda*SpaI, a fish-pathogen-derived SXT-related integrating conjugative element that can mobilize a virulence plasmid. J. Bacteriol. **190:**3353–3361.
36. **Pagani, L., R. Migliavacca, L. Pallecchi, C. Matti, E. Giacobone, G. Amicosante, E. Romero, and G. Rossolini.** 2002. Emerging extended-spectrum β-lactamases in *Proteus mirabilis*. J. Clin. Microbiol. **40:**1549–1552.
37. **Pearson, M., M. Sebaihia, C. Churcher, M. Quail, A. Seshasayee, N. Luscombe, Z. Abdellah, C. Arrosmith, B. Atkin, T. Chillingworth, H. Hauser, K. Jagels, S. Moule, K. Mungall, H. Norbertczak, E. Rabbinowitsch, D. Walker, S. Whithead, N. Thomson, P. Rather, J. Parkhill, and H. Mobley.** 2008. Complete genome sequence of uropathogenic *Proteus mirabilis*, a master of both adherence and motility. J. Bacteriol. **190:**4027–4037.
38. **Pembroke, J., and A. Piterina.** 2006. A novel ICE in the genome of *Shewanella putrefaciens* W3-18-1: comparison with the SXT/R391 ICE-like elements. FEMS Microbiol. Lett. **264:**80–88.
39. **Sidjabat, H., D. Paterson, Z. Qureshi, J. Adams-Haduch, A. O'Keefe, A. Pascual, J. Rodríguez-Baño, and Y. Doi.** 2009. Clinical features and molecular epidemiology of CMY-type β-lactamase-producing *Escherichia coli*. Clin. Infect. Dis. **48:**739–744.
40. **Taviani, E., D. Ceccarelli, N. Lazaro, S. Bani, P. Cappuccinelli, R. Colwell, and M. Colombo.** 2008. Environmental *Vibrio* spp., isolated in Mozambique, contain a polymorphic group of integrative conjugative elements and class 1 integrons. FEMS Microbiol. Ecol. **64:**45–54.
41. **Toma, C., N. Nakasone, T. Song, and M. Iwanaga.** 2005. *Vibrio cholerae* SXT element, Laos. Emerg. Infect. Dis. **11:**346–347.
42. **Verdet, C., V. Gautier, E. Chachaty, E. Ronco, N. Hidri, D. Decré, and G. Arlet.** 2009. Genetic context of plasmid-encoded bla_{CMY-2}-like genes in *Enterobacteriaceae*. Antimicrob. Agents Chemother. **53:**4002–4006.
43. **Waldor, M., H. Tschäpe, and J. Mekalanos.** 1996. A new type of conjugative transposon encodes resistance to sulfamethoxazole, trimethoprim, and streptomycin in *Vibrio cholerae* O139. J. Bacteriol. **178:**4157–4165.
44. **Yagi, T., J. Wachino, H. Kurokawa, S. Suzuki, K. Yamane, Y. Doi, N. Shibata, H. Kato, K. Shibayama, and Y. Arakawa.** 2005. Practical methods using boronic acid compounds for identification of class C β-lactamase-producing *Klebsiella pneumoniae* and *Escherichia coli*. J. Clin. Microbiol. **43:**2551–2558.