

Genomic and Functional Analysis of ICEPdaSpa1, a Fish-Pathogen-Derived SXT-Related Integrating Conjugative Element That Can Mobilize a Virulence Plasmid[∇]

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Integrating conjugative elements (ICEs) are self-transmissible mobile elements that transfer between bacteria via conjugation and integrate into the host chromosome. SXT and related ICEs became prevalent in Asian *Vibrio cholerae* populations in the 1990s and play an important role in the dissemination of antibiotic resistance genes in *V. cholerae*. Here, we carried out genomic and functional analyses of ICEPdaSpa1, an SXT-related ICE derived from a Spanish isolate of *Photobacterium damsela* subsp. *piscicida*, the causative agent of fish pasteurellosis. The ~102-kb DNA sequence of ICEPdaSpa1 shows nearly 97% DNA sequence identity to SXT in genes that encode essential ICE functions, including integration and excision, conjugal transfer, and regulation. However, ~25 kb of ICEPdaSpa1 DNA, including a tetracycline resistance locus, is not present in SXT. Most ICEPdaSpa1-specific DNA is inserted at loci where other SXT-related ICEs harbor element-specific DNA. ICEPdaSpa1 excises itself from the chromosome and is transmissible to other *Photobacterium* strains, as well as to *Escherichia coli*, in which it integrates into *prfC*. Interestingly, the *P. damsela* virulence plasmid pPHDP10 could be mobilized from *E. coli* in an ICEPdaSpa1-dependent fashion via the formation of a cointegrate between pPHDP10 and ICEPdaSpa1. pPHDP10-Cm integrated into ICEPdaSpa1 in a non-site-specific fashion independently of RecA. The ICEPdaSpa1::pPHDP10 cointegrates were stable, and markers from both elements became transmissible at frequencies similar to those observed for the transfer of ICEPdaSpa1 alone. Our findings reveal the plasticity of ICE genomes and demonstrate that ICEs can enable virulence gene transfer.

Integrating conjugative elements (ICEs) are mobile elements that can be excised from their host's chromosome, transfer via conjugation into a new host, and then reintegrate into the chromosome (8, 12). SXT is an ICE that carries multiple antibiotic resistance genes; it was originally discovered in MO10, one of the initial *Vibrio cholerae* O139 clinical isolates from India (35). Prior to the emergence of this novel *V. cholerae* serogroup, SXT was rarely if ever detected in *V. cholerae* O1 isolates (2). Since the emergence and spread of *V. cholerae* O139, ICEs closely related to the MO10 SXT (SXT^{MO10}) have been identified in most *V. cholerae* O1 and O139 clinical isolates from Asia (2, 18, 20, 23). In addition, SXT-related ICEs in African *V. cholerae* O1 isolates (15), as well as in other vibrios (1) and in other *Gammaproteobacteria* (20, 33), have been described previously. SXT is genetically and functionally related to the IncJ element R391, which was derived from a South African *Providencia rettgeri* strain isolated in 1967. Although R391 was initially thought to be an R factor (14), subsequent studies have demonstrated that R391 is an ICE

that is related to SXT (3, 6, 19). The SXT-R391 family of ICEs is now known to have at least 25 members derived from diverse gram-negative organisms and locations (7).

All members of the SXT-R391 family of ICEs encode nearly identical tyrosine recombinases (Int) that mediate the site-specific integration of the elements into *prfC* (7, 22). Another protein, Xis, which acts as a recombination directionality factor, is required in addition to Int for efficient SXT excision (10). The SXT conjugation genes are related to those found in plasmids pCAR1, derived from *Pseudomonas resinovorans* (27), and Rts1, derived from *Proteus vulgaris* (30). The conditions that promote SXT transfer are not fully understood, but it is known that transfer is stimulated by the host SOS response via a pathway that resembles the pathway governing the lytic development of phage lambda (5).

Comparative analyses of the genome sequences of SXT^{MO10} (99.5 kb) and R391 (89 kb) have revealed that these ICEs consist of a conserved set of "backbone" genes that mediate the essential functions of the elements, including their regulation, excision and integration, and conjugative transfer (3, 4, 6). There is more than 95% nucleotide sequence identity between these two elements in the ~65 kb of DNA sequence they share. Both elements contain insertions into this backbone that confer element-specific properties, such as antibiotic resistance. SXT^{MO10} carries genes that mediate resistance to sulfamethoxazole, trimethoprim, chloramphenicol, and streptomycin, whereas R391 mediates resistance to kanamycin and mercury. In some cases, insertions of antibiotic resistance genes appear

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
<i>P. damsela</i> subsp. <i>piscicida</i>		
PC554.2	Virulent strain; <i>prfC</i> ::ICE <i>PdaSpa1</i> Tc ^r	24
DI21	Virulent strain	28
CS31	DI21 derivative; R ^f Kn ^r	32
<i>E. coli</i>		
DH5 α	Cloning strain	Laboratory stock
XL1-Blue MR	$\Delta(mcrA)183 \Delta(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac$	Stratagene
MC1061	F ⁻ <i>araD139</i> $\Delta(ara-leu)7696 galE15 galK16 \Delta(lac)X74 rpsL$ (Sm ^r) <i>hsdR2</i> (r _K ⁻ m _K ⁺) <i>mcrA mcrB1</i>	36
CAG18420	MG1655 <i>lacZU118 lacI42</i> ::Tn10kan	34
BI533	MG1655 Nx ^r	21
BW25113	<i>lacI</i> ^q <i>rrnB</i> _{T14} $\Delta lacZ_{WJ16}$ <i>hsdR514</i> $\Delta araBAD_{AH33}$ $\Delta rhaBAAD_{LD78}$	16
VI499	BW25113 derivative; <i>recA</i>	
CCW026	ICE <i>PdaSpa1</i> exconjugant of MC1061; Sm ^r Tc ^r	This study
CCW01	BW25113 <i>prfC</i> ::ICE <i>PdaSpa1</i>	This study
CCW02	BW25113 <i>prfC</i> ::ICE <i>PdaSpa1</i> Δint	This study
CCW03	VI499 <i>prfC</i> ::ICE <i>PdaSpa1</i>	This study
CCW069	MC1061 transformed with pPHDP10-Cm; Sm ^r Cm ^r	This study
CCW077	CCW026 transformed with pPHDP10-Cm; Sm ^r Tc ^r Cm ^r	This study
CCW088	Nx ^r Tc ^r Cm ^r exconjugant from CCW077 \times BI533	This study
CCW129	Tc ^r Cm ^r Ap ^r exconjugant from PC554.2 \times VI499 transformed with pPHDP10-Cm and with pGG2B; <i>recA</i>	This study
Plasmids		
pSuperCos1	Cosmid cloning vector; Ap ^r Kn ^r	Stratagene
pPHDP10	Native virulence plasmid of <i>P. damsela</i> subsp. <i>piscicida</i> DI21 strain	17
pPHDP10-Cm	pPHDP10 with a Cm ^r cassette from pKD3 inserted in BamHI site; Cm ^r	This study
pKD3	PCR template for one-step chromosomal gene inactivation; Cm ^r	16
pGG2B	<i>setCD</i> genes from SXT _{MO10} cloned into pBAD30; Ap ^r	V. Burrus

to have been mediated by transposons (20). Comparative sequence analyses of SXT^{MO10} and R391 also led to the identification of four sites that appear to correspond to hot spots for the insertion of accessory DNA into these elements (3, 7). These four sites contain totally unrelated sequences in these two ICEs; the recently described ICE*SpuPO1* from *Shewanella putrefaciens* also contains element-specific DNA in three of four of these hot spots (33). Recombination between tandem arrays of ICEs may also contribute to the generation of ICE diversity (7, 9, 11).

Juiz-Rio et al. recently reported that a *Photobacterium damsela* subsp. *piscicida* isolate derived from a diseased sole (*Solea senegalensis*) from a fish farm in Galicia, Spain, appears to contain an SXT-like ICE (24). They found that they could amplify five SXT backbone genes from this virulent *P. damsela* subsp. *piscicida* isolate (PC554.2) and showed that this strain's *prfC* locus contains SXT-related DNA. *P. damsela* subsp. *piscicida* is the causative agent of fish pasteurellosis in both wild and cultured warm-water marine fish (26, 29). The mechanisms of *P. damsela* subsp. *piscicida* pathogenicity are multifactorial and incompletely understood. One of the major virulence factors of *P. damsela* subsp. *piscicida* is the AIP56 toxin, which induces apoptosis in fish neutrophils and macrophages (17). This toxin is encoded in pPHDP10, a 9.6-kb plasmid that is present in most *P. damsela* subsp. *piscicida* strains isolated in Europe. A high-affinity siderophore-mediated iron acquisition system encoded by a genomic island similar to the

Yersinia high-pathogenicity island is also involved in *P. damsela* subsp. *piscicida* virulence (32).

Here, we carried out genomic and functional analyses of the PC554.2-derived ICE. We found that this ICE, dubbed ICE*PdaSpa1*, is indeed closely related to SXT and R391. The ICE*PdaSpa1* genome contained all of the genes known to be required for SXT transfer, excision, and integration. ICE*PdaSpa1* proved to be self-transmissible and to be excised from and integrate into *prfC*. Remarkably, we also found that ICE*PdaSpa1* could mobilize the pPHDP10 virulence plasmid via the formation of an ICE*PdaSpa1*::pPHDP10 cointegrate. Thus, ICE*PdaSpa1* may contribute to the dissemination of virulence genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The strains used in this study are listed in Table 1. *P. damsela* subsp. *piscicida* strains were routinely grown at 25°C in brain heart infusion (BHI; Difco) supplemented with 1% NaCl. *Escherichia coli* strains were routinely grown in Luria-Bertani (LB) broth at 37°C. Bacterial strains were stored frozen at -70°C in LB broth containing 20% (vol/vol) glycerol. Antibiotics were used at the following concentrations: ampicillin, 100 mg liter⁻¹; kanamycin, 50 mg liter⁻¹; rifampin, 50 mg liter⁻¹; tetracycline, 4 mg liter⁻¹ (*Photobacterium* strains) or 12 mg liter⁻¹ (*E. coli* strains); chloramphenicol, 20 mg liter⁻¹; spectinomycin, 100 mg liter⁻¹; streptomycin, 200 mg liter⁻¹; and nalidixic acid, 40 mg liter⁻¹.

DNA preparation and manipulation. Total genomic DNA was prepared with G Nome DNA kits (Q-Biogene). Plasmid and cosmid DNA purification and DNA extraction from agarose gels were accomplished using kits from Qiagen. Recombinant DNA manipulations were carried out by standard procedures. The

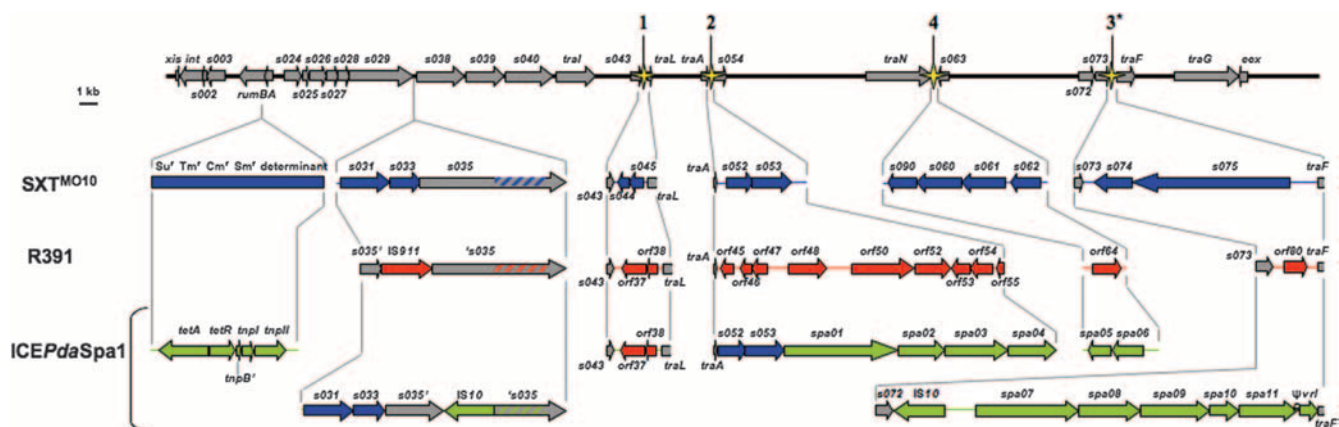


FIG. 1. Schematic representation of portions of the ICEPdaSpa1, SXT^{MO10}, and R391 genomes. Conserved genes are shown as gray arrows, and DNA initially identified in ICEPdaSpa1, SXT^{MO10}, or R391 is shown in green, blue, or orange, respectively. The numbered yellow stars represent the sites of hot spots 1 to 4. Note that the scales used to represent the conserved and the nonconserved genes are different and that some conserved genes have been left out of the figure (as have regions without genes). The ICEPdaSpa1 and SXT^{MO10} insertions in *rumB* are shown in more detail in Fig. 2. The left border of hot spot 3 in ICEPdaSpa1 begins in the 3' end of *s072*, as *s073* is absent from this ICE. In R391 and ICEPdaSpa1, *s035* is split by insertion sequence elements into two ORFs, noted as 's035' and 's035'. The striped sections in *s035* and 's035' correspond to an ICE-specific variable region.

TA cloning kit (Invitrogen) was used for the cloning of PCR products. The deletion of the ICEPdaSpa1 *int* gene was accomplished using the one-step chromosomal gene inactivation technique (16). The ECL direct nucleic acid labeling and detection system (Amersham Biosciences) was used for the Southern blot analyses.

Determination of the ICEPdaSpa1 DNA sequence. To obtain template DNA to determine and assemble the entire ICEPdaSpa1 DNA sequence, we initially constructed a cosmid library from PC554.2 and then isolated a set of overlapping cosmids that hybridized with SXT genes. The cosmid vector SuperCos1 (Stratagene) was used to construct a library of partially Sau3AI-digested DNA isolated from *P. damsela* subsp. *piscicida* PC554.2. The cosmid library was propagated in *E. coli* XLI-Blue MR and screened by colony PCR using primers for the previously described ICEPdaSpa1 *int*, *traI*, *traC*, and *traN* genes (24).

Five cosmids, pCW010, pCW013, pCW017, pCW021, and pCW050, were found to span the complete element. A combination of primers previously used to sequence SXT (4), along with several new primers, enabled us to obtain the complete ICEPdaSpa1 DNA sequence. Automated DNA sequencing was carried out at the Tufts Medical School DNA Sequencing Core Facility. Vector NTI (Invitrogen) was used to assemble DNA sequences. Open reading frames (ORFs) were determined using BioEdit version 7.0.4.1. The FASTA3 and BLAST algorithms were used to assess similarities between the sequences of products of ICEPdaSpa1 DNA and putative protein sequences listed in the European Bioinformatics Institute and NCBI databases.

Bacterial conjugation. Conjugation assays were performed by mixing equal volumes of log-phase cultures of donor and recipient strains. Cell mixtures were concentrated by centrifugation, resuspended in a 0.1 volume of LB or BHI broth, and then applied to 0.45- μ m-pore-size membrane filters (Millipore) on LB or BHI agar plates. Matings were performed for 4 h at 25°C when *P. damsela* subsp. *piscicida* was used either as a donor or as a recipient; the remainder of the matings were performed at 37°C. Cells were collected in 2 ml of LB or BHI broth, and serial dilutions were plated onto the appropriate selective media to determine the numbers of donors, recipients, and exconjugants. The transfer frequency was calculated as the number of exconjugants observed per donor cell.

Real-time quantitative PCR assay for relative quantification of ICEPdaSpa1 *attB*. A real-time quantitative PCR assay was used to measure the percentage of PC554.2 cells that contained unoccupied ICEPdaSpa1 *attB* sites. The amount of *attB* DNA in each sample was normalized to the amount of chromosomal DNA in the sample (determined using a real-time quantitative PCR assay for the *P. damsela* subsp. *piscicida* *tonB* gene). Primer design, reactions, and analysis were performed as described previously (10). Primers to amplify the *attB* region of *P. damsela* subsp. *piscicida* were QattBF (5' TCACGCTAATGTCGAACAGTT ATCA 3') and QattBR (5' GCGTTTCCGAATAATAGAACTTTTTC 3'), and primers to amplify the *tonB* locus were QtonBF (5' CCAAGCCAAAACGCA ATAGC 3') and QtonBR (5' TGACTTCTGCTTTTGCAACATCTT 3').

Analysis of the ICEPdaSpa1 insertion site. PCR assays were used to assess whether *P. damsela* subsp. *piscicida* CS31 or *E. coli* CAG18420 harbored ICEPdaSpa1 in *prfC*. A primer, VISLR (5' GCTGCCATCTTTTATTCTTC 3'), that targets the ICEPdaSpa1 *int* gene was used in combination with a specific primer for the host *prfC* gene. We used primer QattBF (see above) for *P. damsela* subsp. *piscicida* *prfC* and primer EattBF (11) for *E. coli* *prfC*.

Construction of a marked pPHDP10. To construct a marked version of pPHDP10, we first purified the plasmid from *P. damsela* subsp. *piscicida* strain DI21 (17). Plasmid DNA was digested with BamHI, which linearizes pPHDP10, and the 9.6-kb band was gel purified and ligated to a chloramphenicol resistance cassette obtained by PCR amplification of the *cat* gene from the pKD3 plasmid (16). The insertion of the *cat* gene into the BamHI site of pPHDP10 was confirmed by DNA sequencing. The *cat*-marked pPHDP10 was designated pPHDP10-Cm.

Nucleotide sequence accession number. The DNA sequence described in this article has been deposited in the EMBL database under accession number AJ870986.

RESULTS AND DISCUSSION

General organization of the ICEPdaSpa1 genome. Juiz-Rio et al. found that PC554.2, a highly virulent *P. damsela* subsp. *piscicida* isolate, contains homologues of SXT genes that are apparently inserted into *prfC*, the *V. cholerae* SXT integration site, and proposed that these genes are likely to be part of a *P. damsela* subsp. *piscicida* SXT-like ICE (24). We determined the complete DNA sequence of this SXT-like element (here named ICEPdaSpa1) to further comparative genomic analyses of the SXT-R391 family of ICEs. The ICEPdaSpa1 sequence is 102,985 bp in length and contains 86 putative ORFs (Fig. 1). Overall, our assembly and analysis of the complete ICEPdaSpa1 genome sequence confirmed the proposal of Juiz-Rio et al. (24) that this fish pathogen harbors an SXT-R391 family ICE.

The organization of the ICEPdaSpa1 genome proved to be very similar to those of the three other fully sequenced SXT-related ICEs, SXT^{MO10} (4), R391 (6), and ICESpuPO1 (33). All four ICEs consist of a highly conserved core set of genes, encompassing approximately 60 kb (Fig. 1), that mediate crit-

TABLE 2. Predicted products of ICEPdaSpa1-specific genes

Genome region and predicted ORF or product (no. of amino acids)	Positions of ORF in ICEPdaSpa1 ^a	Gene or protein homologue	GenBank accession no. for homologue gene	% Identity/% similarity to homologue ^b
<i>rumB</i> region				
TetA (400)	11676–12878 (compl)	<i>P. damsela</i> subsp. <i>piscicida</i> tetracycline resistance protein, class D	BAA03719	66/78
TetR (200)	12959–13561	<i>E. coli</i> Tet repressor, class D	1A6I	74/85
TnpI (96)	13951–14241	<i>Psychromonas ingrahamii</i> IS3/IS911 transposase	ZP_01350213	62/80
TnpII (243)	14373–15104	<i>Psychromonas ingrahamii</i> integrase	ZP_01350212	64/80
Region between s035' and 's035				
IS10 transposase (402)	28188–29396 (compl)	<i>Salmonella enterica</i> serovar Typhi IS10 transposase	NP_058298	100/100
Hot spot 2				
Spa01 (909)	52041–54770	<i>Psychromonas</i> sp. strain CNPT3 putative helicase	ZP_01215992	40/60
Spa02 (369)	54767–55876	No database matches with significant similarity		
Spa03 (510)	55889–57421	<i>Acidovorax</i> sp. strain JS42 phospholipase D/transphosphatidylase	ZP_01383961	28/47
Spa04 (377)	57736–58869	<i>Desulfovibrio desulfuricans</i> two-component transcriptional regulator, Fis family	ABB37621	38/61
Hot spot 4				
Spa05 (175)	68950–69474 (compl)	No database matches with significant similarity		
Spa06 (257)	69487–70257 (compl)	<i>Salmonella enterica</i> hypothetical protein	AAG03008	38/52
Hot spot 3				
IS10 transposase (402) identical to the IS10 transposase listed above	81904–83112 (compl)	<i>Salmonella enterica</i> serovar Typhi IS10 transposase	NP_058298	100/100
Spa07 (831)	83835–86330	Deltaproteobacterium MLMS-1 heat shock protein Hsp70	EAT04212	80/88
Spa08 (489)	86334–87803	Deltaproteobacterium MLMS-1 AAA ATPase, central region	EAT04211	82/91
Spa09 (553)	87816–89477	Deltaproteobacterium MLMS-1 hypothetical protein	EAT04215	68/82
Spa10 (235)	89470–90177	No database matches with significant similarity		
Spa11 (464)	90286–91680	Deltaproteobacterium MLMS-1 peptidoglycan-binding domain 1	EAT04214	39/51
ψ vrrR	91687–92181	<i>Desulfococcus multivorans</i> vrrR gene	CT009609	88

^a Numbers are nucleotide positions. Compl, complementary strand.

^b Data refer to identity and similarity at the amino acid level, except the value for *vrrR*, which refers to the similarity at the nucleotide sequence level.

ical ICE functions, including regulation, integration and excision, and conjugative DNA processing and transfer, as well as variable numbers of additional genes (Fig. 1) that mediate ICE-specific properties, such as antibiotic resistance. The ICEPdaSpa1 core genes are arranged in the same order as those in the other elements and have 96 to 97% identity to the corresponding SXT and R391 sequences at the nucleotide level. Of the 86 predicted ICEPdaSpa1 ORFs, 69 are also present in SXT^{MO10}, in R391, or in both. ICEPdaSpa1 contains all the genes that are thought to be required for element transfer and regulation (4). Interestingly, s073, a gene previously thought of as a gene common to SXT-related ICEs, is missing in ICEPdaSpa1 (Fig. 1). This gene is apparently not part of the core set of genes required for ICE transfer, since we found that ICEPdaSpa1 is capable of transfer (see below).

Seventeen ICEPdaSpa1 ORFs are reported here for the first time to be present in an SXT-related ICE (Table 2). Nearly all of the ~25 kb of ICEPdaSpa1-specific DNA is found in the same sites that harbor element-specific DNA in other SXT-

related ICEs (Fig. 1). ICEPdaSpa1 contains the same four hot spots (Fig. 1) that exhibit variable DNA sequences in SXT^{MO10} and R391. The two other ICEPdaSpa1 sites that harbor additional ICEPdaSpa1-specific DNA correspond to SXT^{MO10} sites that contain element-specific DNA.

ICEPdaSpa1 antibiotic resistance genes. As SXT^{MO10}, ICEPdaSpa1 contains genes that mediate resistance to antibiotics inserted into the *rumB* locus (Fig. 1 and 2). The *rumB* locus of ICEPdaSpa1 is disrupted at exactly the same base pair as that of SXT^{MO10}, and in contrast, the R391 *rumB* is not disrupted (20). The sequences inserted at *rumB* in ICEPdaSpa1 and SXT are related. The genes at the boundaries of the insertions (*tnp* and s021) in the two ICEs are identical (Fig. 2). The ICEPdaSpa1-specific DNA includes a tetracycline resistance locus that comprises an active (see below) class D *tetA* gene, the *tetR* repressor gene, and two ORFs that appear to encode intact transposases, *tnpI* and *tnpII* (Fig. 2 and Table 2). ICEPdaSpa1 shares additional genetic features with SXT^{MO10} in this region. Interestingly, in both of these ICEs, *tnpB* (or a

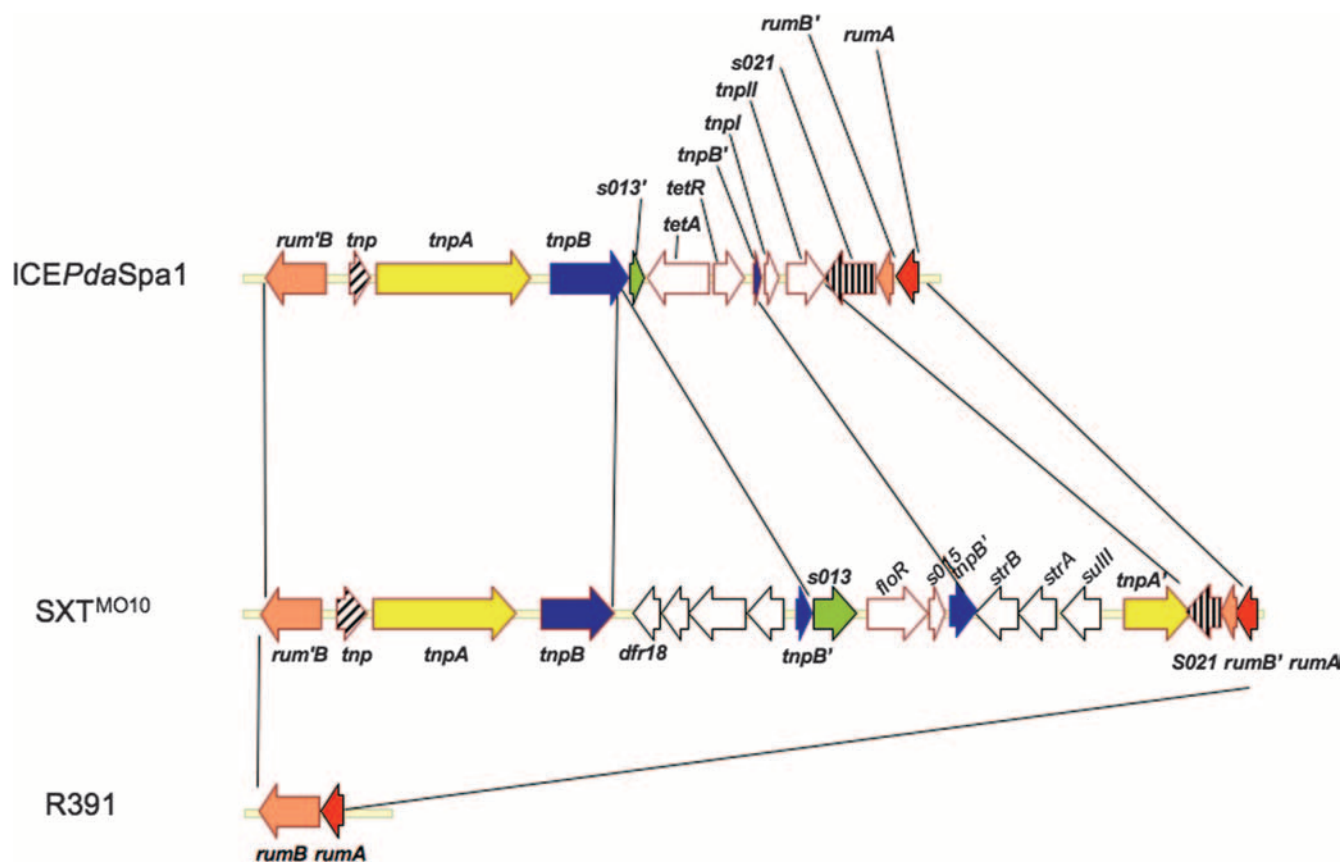


FIG. 2. Comparison of the *rumB* regions in ICEPdaSpa1, SXT^{MO10}, and R391.

tnpB fragment) brackets antibiotic resistance genes, perhaps suggesting that this gene promotes the acquisition of antibiotic resistance genes (Fig. 2). This possibility is reinforced by the finding that *tnpB* or truncated *tnpB* fragments are closely linked to antibiotic resistance genes in other bacterial species and in large *P. damsela* subsp. *piscicida* plasmids (13, 25). Altogether, the structure of the DNA insertion in the *rumB* locus suggests that in this region SXT and ICEPdaSpa1 share a common evolutionary history.

Gene contents of the ICEPdaSpa1 hot spots. As those in other SXT- and R391-related ICEs (3, 9, 33), most of the none-core genes in ICEPdaSpa1 are found in four hot spots (Fig. 1) where SXT-related ICEs appear to acquire additional DNA. Hot spot 1, between *s043* and *traL*, contains two genes, *orf37* and *orf38*, that are also present in this location in R391 but are not found in SXT^{MO10} (Fig. 1). The identity of the contents of hot spots 1 in R391 and ICEPdaSpa1 suggests that these regions of these two ICEs are likely to be derived from a common ancestor. The partial identity of ICEPdaSpa1 to SXT^{MO10} in the *rumB* region and to R391 in hot spot 1 indicates that the ICEPdaSpa1 genome has a mosaic structure and provides further evidence supporting the idea that recombination events occur between ICEs belonging to the SXT-R391 family (7, 11). The content of hot spot 2 in ICEPdaSpa1 also suggests that recombination between SXT-related ICEs shaped ICEPdaSpa1's genome, as this region is composed of sequences found in SXT^{MO10} (*s052* and *s053*) and sequences

unique to ICEPdaSpa1 (*spa01*, *spa02*, *spa03*, and *spa04*) (Fig. 1 and Table 2).

An analysis of the boundaries of hot spot 2 revealed that ICEPdaSpa1, SXT^{MO10}, and R391 share the same nucleotide sequence up to the *traA* stop codon (Fig. 3). At the right end of hot spot 2, downstream of *spa04*, ICEPdaSpa1 lacks a 19-bp sequence present in SXT^{MO10} and R391 (Fig. 3) that was previously described as the 3' boundary of hot spot 2 (3). This 19-bp sequence was presumably deleted during the acquisition of the novel sequences found in ICEPdaSpa1's hot spot 2.

The boundaries and content of hot spot 3 in ICEPdaSpa1 differ from those in SXT^{MO10} and R391 (Fig. 1 and 3; Table 2). The 5' ends of the corresponding hot spots in the latter two ICEs are located downstream of *s073*; however, this gene is not present in ICEPdaSpa1. The 5' boundary of ICEPdaSpa1's hot spot 3 is found just downstream from *s072* (Fig. 1 and 3), and the 3' boundary is 27 bp downstream of the corresponding boundaries found in SXT^{MO10} and R391. Since *s073* is not present in ICEPdaSpa1 but this ICE is functional (see below), *s073* should not be considered to be a part of the functional core set of genes in SXT-related ICEs. The new locations of the hot spot 3 boundaries in ICEPdaSpa1 may have arisen from the insertion of the *IS10*-like transposase gene found in this region (Table 2). Aside from the *IS10*-like sequence, the predicted proteins encoded in ICEPdaSpa1's hot spot 3 have similarity to Hsp70 and an AAA ATPase, as well as to hypothetical proteins of unknown functions (Table 2). Interestingly,



FIG. 3. DNA sequences found at the boundaries of the four hot spots in ICEPdaSpa1, SXT_{MO10}, and R391. Conserved DNA is shown in gray, ICEPdaSpa1-specific DNA is shown in green, SXT-specific DNA is shown in blue, and R391-specific DNA is shown in orange. Nucleotide differences in the conserved DNA are indicated in black. The box represents the stop codon in *traA*.

the 3' end of the ICEPdaSpa1 hot spot 3 DNA, between *spa11* and *traF*, contains 495 bp of an apparently noncoding sequence that is 88% identical to a portion of the so-called *vrl* island of *Desulfococcus multivorans* at the nucleotide level (Fig. 1; Table 2).

ICEPdaSpa1's hot spot 4, between *traN* and *s063*, contains two genes whose predicted protein products have unknown functions (Fig. 1 and Table 2). Although the contents of hot spots 4 in ICEPdaSpa1 and R391 differ, the 5' ends of these hot spots are nearly identical (Fig. 3).

ICEPdaSpa1 is excised from the chromosome and can be conjugally transferred to other bacteria. The known SXT-related ICEs can be excised from the chromosomes of their respective hosts to become extrachromosomal circular forms. We tested whether ICEPdaSpa1 is excised from the PC554.2 chromosomal *prfC* locus by using a PCR assay that detects the unoccupied *prfC* locus. This assay, which relies on primers that flank the ICEPdaSpa1 integration site, revealed an ~120-bp product when PC554.2 DNA was used as the template, suggesting that ICEPdaSpa1 was excised from the chromosomes in a fraction of the cells in the culture, yielding *attB* sites. Using a real-time quantitative PCR assay (10), we determined that ICEPdaSpa1 was excised from approximately 0.4% of cells in an overnight culture, a percentage that is similar to that observed for SXT^{MO10} excision from the *V. cholerae* chromosome (10).

Plate mating assays were used to test whether ICEPdaSpa1 could be transferred to other *P. damsela* subsp. *piscicida* strains, as well as to *E. coli*. These assays were facilitated by our discovery that the ICEPdaSpa1 genome contains a *tet* locus (see above) and, thus, we could use tetracycline to select for

exconjugants containing ICEPdaSpa1. When we carried out mating experiments with PC554.2 and CS31, a rifampin-resistant (R^f) and kanamycin-resistant (Kn^r) *P. damsela* subsp. *piscicida* strain lacking ICEPdaSpa1, tetracycline-resistant (Tc^r) R^f Kn^r exconjugants were obtained at a frequency of 1.3×10^{-4} exconjugants/donor (Table 3). Using a PCR-based assay (see Materials and Methods), we found that all 50 exconjugants tested contained ICEPdaSpa1 integrated into the *prfC* gene of the recipient. ICEPdaSpa1 could also be transferred from PC554.2 to *E. coli* K-12 strains CAG18420 and MC1061, though at somewhat lower frequencies than that of the transfer to *P. damsela* subsp. *piscicida* CS31 (Table 3 and data not shown). All *E. coli* exconjugants tested contained ICEPdaSpa1 integrated into the *E. coli prfC* gene. Finally, *E. coli* ICEPdaSpa1 exconjugants could serve as donors to transfer ICEPdaSpa1 to other *E. coli* strains at high frequencies (Table 3); in the latter *E. coli* exconjugants, ICEPdaSpa1 was integrated at *prfC*. Together, these observations demonstrate that ICEPdaSpa1 is a functional, self-transmissible ICE capable of conjugal transfer and integration into and excision from the chromosome.

***int* and *recA* are critical for ICEPdaSpa1 transfer.** The SXT^{MO10} integrase is crucial for SXT^{MO10} transmission since this recombinase is essential for the element's excision and integration (22). As expected, the ICEPdaSpa1 *int*, which is nearly identical to the SXT^{MO10} *int*, proved to be critical for ICEPdaSpa1 transfer. The deletion of *int* from ICEPdaSpa1 reduced the frequency of transfer of this element by nearly 5 orders of magnitude (Table 3, compare lines 3 and 4). The very low frequency of transfer of the ICEPdaSpa1 *int* may be attributable to the Hfr-like transfer of the ICEPdaSpa1 markers

TABLE 3. ICEPdaSpa1 is transmissible

Donor strain name ^a	Donor strain description	Recipient strain name	Recipient strain description	Transfer frequency ^b
PC554.2	<i>P. damsela</i>	CS31	<i>P. damsela</i>	1.3×10^{-4}
PC554.2	<i>P. damsela</i>	CAG18420	<i>E. coli</i> ; Kn ^r	1.5×10^{-5}
CCW01	<i>E. coli</i> BW25113 <i>prfC</i> ::ICEPdaSpa1	CAG18420	<i>E. coli</i> ; Kn ^r	2.2×10^{-3}
CCW02	<i>E. coli</i> BW25113 <i>prfC</i> ::ICEPdaSpa1Δ <i>int</i>	CAG18420	<i>E. coli</i> ; Kn ^r	4.1×10^{-8}
CCW03	<i>E. coli</i> BW25113 <i>recA prfC</i> ::ICEPdaSpa1	CAG18420	<i>E. coli</i> ; Kn ^r	3.7×10^{-5}

^a All donor strains harbored ICEPdaSpa1.

^b Transfer frequencies were calculated as the numbers of exconjugants divided by the numbers of donors. The values shown are the means of results from three independent assays.

TABLE 4. ICE*PdaSpa1* enables the transfer of pPHDP10

Mating	Donor strain	Relevant property(ies) of donor strain	Frequency of transfer of following selected marker into exconjugants ^{a,b} :		% Of exconjugants with cotransfer of following unselected marker:	
			Cm ^r	Tc ^r	Tc ^r	Cm ^r
1	<i>E. coli</i> CCW069	pPHDP10-Cm	<1.0 × 10 ^{-9c}	NA ^d	NA	NA
2	<i>E. coli</i> CCW077	pPHDP10-Cm ICE <i>PdaSpa1</i>	1.3 × 10 ⁻⁸	1.7 × 10 ⁻³	100	<0.01 ^c
3	<i>E. coli</i> CCW088	Exconjugant from mating 2 (Cm ^r Tc ^r)	2.6 × 10 ⁻³	2.9 × 10 ⁻³	100	100

^a The plasmid pPHDP10-Cm encodes resistance to chloramphenicol (Cm^r) and ICE*PdaSpa1* encodes resistance to tetracycline (Tc^r).

^b Transfer frequencies were calculated as the numbers of exconjugants with the indicated marker divided by the numbers of donors. The values shown are the means of results from at least three independent assays; standard errors were ≤20%.

^c Assay limit.

^d NA, not applicable.

as previously observed with SXT^{MO10} (21). Similarly, given the identity of the predicted amino acid sequences of the ICE*PdaSpa1* and SXT^{MO10} SetR repressors, we expected that *recA* would be important for ICE*PdaSpa1* transfer. *RecA* is thought to promote the autocleavage of SetR, thereby alleviating its repression of *setDC*, the activators of SXT^{MO10} transfer (5). The frequency of the transfer of ICE*PdaSpa1* from a *recA* *E. coli* donor was nearly 2 orders of magnitude lower than the frequency of transfer from an isogenic *recA*⁺ *E. coli* donor (Table 3). This observation suggests that the regulation of ICE*PdaSpa1* transfer is similar to that of SXT^{MO10} transfer.

ICE*PdaSpa1* can mobilize a virulence plasmid and acquire new DNA. Some *P. damsela* subsp. *piscicida* strains, including PC554.2, harbor pPHDP10, an ~10-kb plasmid that encodes an important *P. damsela* subsp. *piscicida* virulence factor, AIP56. This toxin induces apoptosis in fish macrophages and neutrophils (17). We constructed pPHDP10-Cm, a pPHDP10 derivative containing a chloramphenicol resistance cassette, to test whether *E. coli* harboring the ICE*PdaSpa1* element could mobilize this virulence plasmid. Isogenic *E. coli* strains CCW069 (MC1061 pPHDP10-Cm) and CCW077 (MC1061 *prfC*::ICE*PdaSpa1* pPHDP10-Cm) were used as donors in conjugation experiments, and BI533, a nalidixic acid-resistant (Nx^r) derivative of *E. coli* K-12 strain MG1655, was used as a recipient. While no exconjugants were detected when CCW069 was used as a donor, the marked virulence plasmid was transferred at a low but reproducible frequency (~1.3 × 10⁻⁸ exconjugants/donor) from CCW077 (Table 4). Given this low frequency of pPHDP10-Cm transfer, we were concerned that the apparent Nx^r chloramphenicol-resistant (Cm^r) exconjugants were donors that had become spontaneously resistant to nalidixic acid; however, we excluded this possibility by identifying additional markers characteristic of the recipient cells in all exconjugants tested (data not shown). Thus, ICE*PdaSpa1* can enable the horizontal transmission of pPHDP10-Cm. All of the Cm^r exconjugants containing pPHDP10-Cm also contained ICE*PdaSpa1* and were resistant to tetracycline (Table 4), suggesting that pPHDP10-Cm transfer depends on the cotransfer of ICE*PdaSpa1* from the donor and not merely the presence of ICE*PdaSpa1* in the donor. The transfer of ICE*PdaSpa1* did not appear to be influenced by the presence of pPHDP10-Cm in donor cells. The frequency of ICE*PdaSpa1* transfer from CCW077 was ~1.7 × 10⁻³ exconjugants/donor, similar to the frequency of ICE*PdaSpa1* transfer from an *E. coli* donor lacking pPHDP10-Cm (Table 3) and ~5 orders of

magnitude greater than the frequency of the transfer of pPHDP10-Cm (Table 4).

Since there was strict linkage of pPHDP10-Cm transfer to the transfer of ICE*PdaSpa1*, we speculated that pPHDP10-Cm and ICE*PdaSpa1* formed a cointegrate at some point prior to or during transfer from the donor to the recipient. In support of this idea, we found that pPHDP10-Cm was not recoverable as a plasmid from exconjugants derived from the mating of CCW077 with BI533, even though this plasmid was detectable in the donor (data not shown). To further explore this possibility, we tested whether exconjugants that contained both pPHDP10-Cm and ICE*PdaSpa1* transferred both elements at similar frequencies. One of the BI533-derived Nx^r Cm^r Tc^r exconjugants (designated CCW088) isolated as described above was used as a donor of ICE*PdaSpa1* and pPHDP10-Cm in plate mating experiments with *E. coli* CAG18420 (Kn^r) as the recipient. In these experiments, we independently selected for the transfer of ICE*PdaSpa1* (yielding Tc^r Kn^r exconjugants) and the transfer of pPHDP10-Cm (yielding Cm^r Kn^r exconjugants). Remarkably, the frequency of the transfer of pPHDP10-Cm (2.6 × 10⁻³ exconjugants/donor) was approximately the same as the frequency of the transfer of ICE*PdaSpa1* (2.9 × 10⁻³ exconjugants/donor) (Table 4). The similar transfer frequencies of ICE*PdaSpa1* and pPHDP10-Cm from CCW088 were in marked contrast to what we observed when the original host of these two elements (CCW077) was tested as the donor (Table 4). Using replica plating, we found that 100% of Tc^r exconjugants (containing ICE*PdaSpa1*) were also Cm^r (i.e., contained pPHDP10-Cm) and that 100% of Cm^r exconjugants (containing pPHDP10-Cm) were also Tc^r (i.e., contained ICE*PdaSpa1*) (Table 4), establishing that pPHDP10-Cm and ICE*PdaSpa1* were cotransferred from CCW088. PCR analyses verified that for all exconjugants tested, Cm^r colonies contained additional pPHDP10-Cm sequences (including the gene encoding the AIP56 toxin) and all Tc^r colonies contained additional ICE*PdaSpa1* sequences. Taken together, these observations provide strong evidence that in CCW088, pPHDP10-Cm is integrated into ICE*PdaSpa1*. Thus, independent selection for the transfer of either element invariably results in the cotransfer of the two elements. Independently isolated Cm^r Nx^r exconjugants from the CCW077 × BI533 mating also harbored ICE*PdaSpa1*::pPHDP10-Cm cointegrates (data not shown).

The integration of pPHDP10-Cm into ICE*PdaSpa1* might have been expected to compromise this ICE's transmissibility,

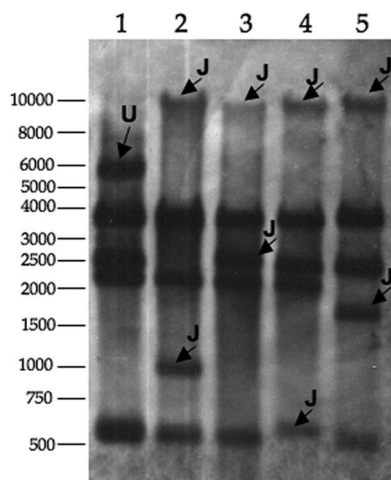


FIG. 4. Southern blot analysis of chromosomal DNA isolated from four independently derived Cm^r Tc^r Kn^r exconjugants from CCW088 \times CAG18420 matings by using pPHDP10-Cm DNA as a probe. Lane 1, SacI-digested pPHDP10-Cm (the U refers to undigested plasmid DNA); lanes 2 to 5, SacI-digested DNA from four Cm^r Tc^r Kn^r exconjugants. The band patterns observed for the four exconjugants reveal that the pPHDP10-Cm integration site in each exconjugant differs from those in the other exconjugants; the J's mark junction fragments. Numbers at the left are molecular weight markers.

but this was not the case. The frequencies of the transfer of hybrid ICEs from CCW088 were similar to the frequencies of the transfer of ICEPdaSpa1 alone from CCW077 and from other donors (data not shown). Finally, the ICEPdaSpa1::pPHDP10-Cm hybrid was found to integrate into *prfC*. Thus, the integration of pPHDP10-Cm into ICEPdaSpa1 yielded a functional hybrid ICE capable of disseminating a potent toxin.

We analyzed the sites of pPHDP10-Cm integration in four independently isolated Cm^r Tc^r Kn^r exconjugants to begin to address the mechanism of ICEPdaSpa1::pPHDP10-Cm cointegrate formation. For these experiments, SacI-digested pPHDP10-Cm was used as a probe to investigate whether this plasmid was integrated into the same or different locations in the chromosomes of the four exconjugants. The probe hybridized to the four SacI pPHDP10-Cm restriction fragments of the correct predicted sizes (Fig. 4, lane 1). The bands detected in the SacI-digested DNA from the four exconjugants revealed that pPHDP10-Cm was integrated into the chromosome. In the DNA from each of the exconjugants, one of the bands observed in the SacI-digested pPHDP10-Cm DNA was absent and two new bands (junction fragments) were apparent (Fig. 4, lanes 2 to 5). The junction fragments in each exconjugant differed from those in the other exconjugants, indicating that the site of pPHDP10-Cm integration differed in each exconjugant. Thus, pPHDP10-Cm does not integrate into ICEPdaSpa1 in a site-specific fashion. In one of the exconjugants, we cloned the pPHDP10-Cm chloramphenicol resistance gene along with adjacent DNA. The DNA sequence of this cloned fragment revealed that pPHDP10-Cm was integrated into *s040*, an ICEPdaSpa1 gene of unknown function. Previous studies have revealed that *s040* is not required for SXT^{MO10} excision, integration, or transfer (4).

We introduced pPHDP10-Cm into a *recA* derivative of MC1061 (yielding strain CCW129) to explore if homologous re-

combination was required for the formation of ICEPdaSpa1::pPHDP10-Cm cointegrates. Since *recA* plays a critical role in promoting ICEPdaSpa1 transfer, for these experiments a *setCD* expression vector, pGG2B, was introduced into CCW129 to bypass the role of *recA* in ICEPdaSpa1 transfer (5). Remarkably, the marked virulence plasmid was transferred at approximately the same frequencies from CCW129 (5×10^{-7} exconjugants/recipient) and from CCW077 (5×10^{-7} exconjugants/recipient). (Since the overexpression of *setCD* is toxic for the donors, for these experiments, transfer frequencies are expressed as numbers of exconjugants per recipient rather than per donor.) As described above for exconjugants derived from CCW077 donors, all exconjugants derived from CCW129 donors were resistant to tetracycline as well as to chloramphenicol, suggesting that ICEPdaSpa1::pPHDP10-Cm cointegrates had formed. Additional pPHDP10-Cm sequences besides the Cm^r gene were detected in the exconjugants, providing additional evidence that cointegrates had formed. These observations indicate that *recA* is not required in the formation of ICEPdaSpa1::pPHDP10-Cm cointegrates. As ICEPdaSpa1 and pPHDP10-Cm do not show long regions of nucleotide identity, it is possible that the insertion sequences in pPHDP10-Cm and/or ICEPdaSpa1 promote cointegrate formation. Alternatively, the ICEPdaSpa1 homologues of the phage lambda *bet* and *exo* genes may facilitate the formation of cointegrates.

Summary and conclusions. Our findings show that there is considerable conservation in the genomes of SXT-related ICEs. The ICEPdaSpa1 genome is the fourth sequenced SXT-related ICE genome. The sequenced ICEs are derived from four different *Gammaproteobacteria*: *P. damsela* subsp. *piscicida* (ICEPdaSpa1), *V. cholerae* (SXT^{MO10}), *Providencia rettgeri* (R391), and *Shewanella putrefaciens* (ICESpuPO1). They all contain a set of highly conserved genes that are required for essential element functions, including genes for regulation (such as *setR* and *setDC*), excision and integration (*xis* and *int*), and conjugative functions (14 *tra* genes). These conserved core genes are arranged in the same order in the different ICEs.

Our findings also highlight the considerable plasticity of the genomes of SXT-related ICEs. Although we found that the ICEPdaSpa1 genome contains nearly all of the conserved genes present in SXT^{MO10} and R391, this fish-pathogen-derived ICE also harbors 25 kb of DNA that is not found in other SXT-related ICEs. Nearly all of the ICEPdaSpa1-specific DNA is found in the same locations as the ICE-specific DNA found in SXT^{MO10} and R391. The conservation of the insertion sites may simply reflect that these sites can accommodate additional DNA without altering ICE function. However, it is also possible that there are specific mechanisms that promote the integration of foreign DNA at these sites. Recombination between SXT-related ICEs appears to be another mechanism for generating diversity in this group of mobile elements.

Remarkably, the ICEPdaSpa1 genome remains plastic. We found that ICEPdaSpa1 could mobilize a *P. damsela* subsp. *piscicida* virulence plasmid via the formation of a cointegrate. The transfer of this new ICEPdaSpa1::pPHDP10-Cm hybrid ICE did not appear to be compromised by the addition of pPHDP10-Cm, likely reflecting the fact that we identified hybrids based on their capabilities for exconjugant formation. Plasmid pPHDP10-Cm integrated into ICEPdaSpa1 in a non-site-specific fashion independently of RecA. Future studies

to elucidate the mechanism of pPHDP10-Cm::ICE*PdaSpaI* cointegrate formation should yield important insights into processes that govern ICE evolution. Previous work has revealed that SXT-related ICEs can mobilize chromosomal DNA in an Hfr-like manner (21, 31) and that SXT can mobilize a plasmid in *trans* (21). Our present findings suggest that SXT-related ICEs can still acquire DNA, an additional mechanism whereby these mobile elements can promote horizontal gene flux.

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