Formation of Chromosomal Tandem Arrays of the SXT Element and R391, Two Conjugative Chromosomally Integrating Elements That Share an Attachment Site

BIANCA HOCHHUT,[1,2] JOHN W. BEABER,[1] ROGER WOODGATE,[3] AND MATTHEW K. WALDOR[1,2]*

Division of Geographic Medicine/Infectious Diseases, New England Medical Center and Tufts University School of Medicine,[1] and Howard Hughes Medical Institute,[2] Boston, Massachusetts 02111, and Section on DNA Replication, Repair and Mutagenesis, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892[3]

The SXT element, a conjugative, self-transmissible, integrating element (a constin) originally derived from a *Vibrio cholerae* O139 isolate from India, and IncJ element R391, originally derived from a South African *Providencia rettgeri* isolate, were found to be genetically and functionally related. Both of these constins integrate site specifically into the *Escherichia coli* chromosome at an identical attachment site within the 5′ end of *prfC*. They encode nearly identical integrases, which are required for chromosomal integration, excision, and extrachromosomal circularization of these elements, and they have similar *tra* genes. Therefore, these closely related constins have virtually identical mechanisms for chromosomal integration and dissemination. The presence of either element in a recipient cell did not significantly reduce its ability to acquire the other element, indicating that R391 and SXT do not encode surface exclusion determinants. In cells harboring both elements, SXT and R391 were integrated in tandem fashion on the chromosome, and homologous recombination appeared to play little or no role in the formation of these arrays. Interference between R391 and SXT was detected by measuring the frequency of loss of an unselected resident element upon introduction of a second selected element. In these assays, R391 was found to have a stronger effect on SXT stability than vice versa. The level of expression and/or activity of the donor and recipient integrases may play a role in the interference between these two related constins.

Similar genetic elements tend not to coexist within the same host cell. Instead, related elements of the same class usually "repel" one another in some fashion. For different types of genetic elements, the molecular bases of incompatibility differ. Plasmid incompatibility, for example, is generally mediated by competition for replication and/or partitioning systems (19). Conjugative plasmids also frequently inhibit host cell entry of related plasmids by altering the host cell's surface (1). Similarly, some bacteriophages alter the surface of host cells to exclude other phages. Phages can also prevent the replication of similar phages through immunity mechanisms (25). Thus, both plasmid and phage incompatibility can depend either on preventing entry of new DNA into a potential host or on inhibiting the replication of new DNA after it has breached the host cell barrier.

Chromosomally integrating mobile genetic elements that are transferred between cells via conjugation—often referred to as conjugative transposons—have been found with increasing frequency in both gram-negative and gram-positive bacteria. Unlike the case for phages and plasmids, relatively little is known about whether similar conjugative transposons can coexist within the same host cell. The well-studied conjugative transposon Tn916, which does not integrate site specifically, can be present in more than one copy at different sites in the host cell chromosome, and the presence of Tn916 in a recipient cell does not inhibit acquisition of a second copy (18, 26).

We previously identified a conjugative transposon-like element, the SXT element, encoding resistance to sulfamethoxazole (Su[r]), trimethoprim (Tm[r]), chloramphenicol (Cm[r]), and streptomycin (Sm[r]) in the recently emerged O139 serogroup of *Vibrio cholerae* (30). The SXT element (henceforth referred to as SXT), or very closely related elements, have subsequently been detected in all O1 and O139 *V. cholerae* clinical isolates from the Indian subcontinent (11). In the laboratory, this element can be transferred between *V. cholerae* strains as well as to a number of other gram-negative bacterial species. An autonomously replicating extrachromosomal form of SXT has not been detected; however, an extrachromosomal circular form of the element has been observed and is thought to be an intermediate in its transfer (11). Formation of this extrachromosomal circular form of SXT requires the SXT-encoded site-specific recombinase (Int), which is closely related to the integrases found in lambdoid bacteriophages. Similar to these phages, SXT integrates site specifically into the chromosome in an *int*-dependent, *recA*-independent fashion via recombination between element (*attP*) and chromosomal (*attB*) sequences that are nearly identical. The SXT chromosomal attachment site is within the 5′ end of *prfC*, which encodes protein chain release factor 3 (11). Since integration of SXT resembles phage integration more than transposition, we thought that the term "conjugative transposon" would not be an informative classification for this element. Instead, we proposed a new

* Corresponding author. Mailing address: Division of Geographic Medicine/Infectious Diseases, New England Medical Center and Tufts University School of Medicine, NEMC 041, 750 Washington St., Boston, MA 02111. Phone: (617) 636-7618. Fax: (617) 636 5292. E-mail: mwaldor@lifespan.org.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Reference or construction
E. coli K-12		
CAG18439	MG1655 *lacI42*::Tn*10*	27
HW220	CAG18439 *prfC*::SXT	11
BI554	HW220 Δ*int*	10
JO99	CAG18439 *prfC*::R391	This study
JO113	CAG18439 *prfC*::R391-SXT	Kn^r SXT^r exconjugant from JO100 × HW220
JO115	CAG18439 *prfC*::R391-SXT	Kn^r SXT^r exconjugant from BI537 × JO99
JO116	CAG18439 *prfC*::SXT-R391	Kn^r SXT^r exconjugant from BI537 × JO99
BI533	MG1655 Nal^r	10
BI537	BI533 *prfC*::SXT	This study
JO100	BI533 *prfC*::R391	This study
JO162	JO100 Δ*int*	This study
KB1	MG1655 *recA56 gutA52 gutR*::Tn*10*	K. Bettenbrock, unpublished data
BI616	KB1 *prfC*::SXT	This study
JO139	KB1 *prfC*::R391	This study
BI742	KB1 *prfC*::SXT-R391	Kn^r SXT^r exconjugant from BI537 × JO139
BI743	KB1 *prfC*::R391-SXT	Kn^r SXT^r exconjugant from BI537 × JO139
BI844	KB1 *prfC*::R391-SXT	Kn^r SXT^r exconjugant from JO100 × BI616
BI845	KB1 *prfC*::SXT-R391	Kn^r SXT^r exconjugant from JO100 × BI616
RW96-R391	Δ(*umuDC*)595::*cat prfC*::R391	9
Plasmids		
pGB2	Spc^r pSC101 derivative	5
pRLH421	22.5-kbp extrachromosomal R391 DNA cloned into pGB2, Spc^r Kn^r	9
pCRII	Cloning vector, Ap^r Kn^r	Invitrogen
pINT6	pCRII *int*$_{R391}$$^+$	This study
pINT391-19	pUC19 *int*$_{R391}$$^+$	This study
pINT18	pUC19 *int*$_{SXT}$$^+$	This study

term, constin, an acronym for SXT's properties (conjugative, self-transmissible, and integrating) to classify this and other elements with similar features (11).

The conjugative element R391, which mediates resistance to kanamycin (Kn^r) and mercury (Hg^r), was isolated in Pretoria, South Africa, in 1967 from a strain originally classified as *Proteus rettgeri* (6) but subsequently reclassified as *Providencia rettgeri* (23). R391 was initially described as an R plasmid, because its antibiotic resistance genes could be transferred to other strains by conjugation (6). Since R391 could coexist with plasmids of all known incompatibility (Inc) groups, it was assigned to a new Inc group, IncJ (6). Since its first description in the early 1970s, only a few other members of the IncJ group have been described, although these elements have been derived from diverse species and geographic locations. Examples include R997, derived from an Indian isolate of *Proteus mirabilis* and carrying Ap^r, Su^r, and Sm^r determinants (14), pMERPH (Hg^r), isolated in England from *Shewanella putrefaciens* (24), and pJY1, isolated in the Philippines from *V. cholerae* El Tor (32). Like SXT, pJY1 mediates Su^r, Sm^r, and Cm^r. Although these elements were originally classified as plasmids, all attempts to isolate extrachromosomal DNA from strains containing these elements have failed, with one notable exception, in which small amounts of extrachromosomal DNA were detected (9). Instead, R391 was detected within the *Escherichia coli* chromosome (16, 20) and the integration site was localized by classical Hfr mapping techniques between *uxuA* (98 min on the *E. coli* map) and *serB* (99.5 min) (15). Based on these findings, Murphy and Pembroke reclassified R391 as a conjugative transposon (16).

Although IncJ elements were collectively assigned to a plasmid incompatibility group based on their compatibility with other plasmids, these elements do not exhibit classic plasmid incompatibility toward each other. That is, the presence of an IncJ element in a recipient cell does not reduce the frequency of transfer of an additional IncJ element in conjugation assays. Instead, incompatibility in the IncJ group has been assessed by measuring the frequency of loss of an unselected resident element upon introduction of a second selected element in conjugation assays (8, 24). In a recent investigation (22), Pembroke and Murphy studied the incompatibility of the two IncJ elements R391 and R997. They observed that loss of markers of the nonselected element was highly reduced in a *recA* mutant strain and concluded that R391 and R997 recombine to form hybrid elements carrying either the resistance genes of both original elements or only those which were selected for. Also, in *recA* recipient cells already harboring either R391 or R997, they were able to isolate an extrachromosomal form of the introduced element. Their observations suggested that when the chromosomal attachment site is occupied by an IncJ element, a newly introduced element either integrates into the chromosome by homologous recombination with the resident element or remains extrachromosomal in *recA* mutant strains. In this report, we show that R391 is closely related to SXT and that the interactions between R391 and SXT fundamentally differ from those described for R391 and R997. Cells harboring both SXT and R391 contain tandem arrays of these elements, and incompatibility between these two constins seems to be mediated by the activity of their *int* genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are described in Table 1. RW96 harboring R391 (9) was used as a donor for R391. Bacterial strains were routinely grown in Luria-Bertani (LB)

broth (3). Bacterial strains were maintained 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}; sulfamethoxazole, 160 mg liter^{-1}; trimethoprim, 32 mg liter^{-1}; tetracycline, 10 mg liter^{-1}; and nalidixic acid, 40 mg liter^{-1}.

To construct pINT6, a 1.8-kbp fragment was amplified with primers XIS5 (5′-TGCGTGACGAAAGCATCAATG-3′) and INT4 (5′-CTTCGCCAGAGC GTCGTATA-3′), with chromosomal DNA isolated from JO99 as template. The product was then cloned into the vector pCRII by using the Topo TA cloning kit (Invitrogen, Carlsbad, Calif.) as specified by the manufacturer. pINT391-19 is a pUC19 derivative with int_{R391} under the control of $lacZ_P$, and pINT18 is a pUC18 derivative with int_{SXT} under the control of the same promoter. An int derivative of R391 in JO100 was constructed by a similar procedure to that described previously for SXT (10). In the resulting strain, JO162, bp 305 to 1025 of int are deleted.

Bacterial conjugations and incompatibility tests. Conjugation experiments were routinely carried out as described previously (30). To compare the transfer frequency of matings performed on solid surfaces or in broth, donor and recipient cells were initially grown to mid-log phase in LB medium. Donor and recipient cells were mixed in a 1:1 ratio and incubated for 2 h in either 2 ml of LB broth or on LB agar plates. Subsequently, dilutions were spread on selective media to determine the number of exconjugants and donor and recipient cells. The frequency of transfer was calculated by dividing the number of SXT^r or Kn^r exconjugants, respectively, by the number of donor cells. To assess the incompatibility between R391 and SXT, each element was transferred into a recipient strain that already carried the other element. In these conjugation assays, only the incoming element was selected; the resident element was not subject to selection. Finally, 100 exconjugants from each mating were tested for the presence of markers characteristic of the original element. Incompatibility was calculated as the percent loss of the nonselected element.

Molecular biology procedures. Plasmid DNA was prepared using the Qiaprep Spin Miniprep kit (Qiagen, Valencia, Calif.), and chromosomal DNA was isolated with the Genome DNA kit (Bio 101, Vista, Calif.) as described by the manufacturer. Recombinant DNA manipulations were carried out by standard procedures (3). Southern blotting was performed with probes conjugated to horseradish peroxidase to enable hybridization to be detected with a chemiluminescent substrate (Amersham) as described previously (28). Automated DNA sequencing was carried out as described previously (29) at the Tufts Medical School DNA Sequencing Core Facility. Computer analysis of DNA sequences was performed with the MacVector and AssemblyLIGN programs (Oxford Molecular Group, Campbell, Calif.) and the BLAST programs (2), available on the website of the National Center for Biotechnology Information, Bethesda, Md.

DNA sequence analysis of the extrachromosomal form of R391. Ho et al. (9) isolated small amounts of extrachromosomal R391 DNA from a $recA718$ strain, RW96. This extrachromosomal R391 DNA was partially digested with EcoRI and cloned into the low-copy-number vector pGB2 (5) by selecting for the R391-encoded kanamycin resistance gene. Several isolates were obtained that contained one or more EcoRI fragments. One of these, called pRLH421, contained an insert of ~22.5 kb comprising four R391 EcoRI fragments of ~11, 8, 3, and 0.5 kb and was chosen for further study. The DNA sequence 3′ to the $rumAB$ operon (13) was obtained using standard "primer-walking" sequencing protocols by Lark Technologies (Houston, Tex). The sequence has been deposited in GenBank under accession number U13633.

PCR assays for detection of R391 integration into $prfC$ in $E. coli$ K-12 and for detection of the circular extrachromosomal form of R391. Amplification of the chromosome-R391 junction fragments was carried out as described previously for SXT using primers 3, 6, 7, and 8 (11). Primers which are oriented toward the left and right SXT-chromosome junctions (primers 4 and 5 in reference 11) were used for detection of a circularized, extrachromosomal form of R391. These PCR assays were carried out using overnight cultures as template DNA as described previously (11).

Determination of the order of tandemly arranged R391 and SXT. The leftmost element-chromosome junction in tandemly arranged R391 and SXT elements was assayed by PCR using the previously described primers 6 and 4 (11). Using these primers, integration of SXT into $attL$ resulted in a 0.8-kbp product while integration of R391 into $attL$ yielded a 2.8-kbp product. DNA corresponding to the rightmost element-chromosome junction was amplified using primer 8 (11) and primer 9 (5′-ATAGACTGAAGTGCTTGCGG-3′). The resulting 3.0-kbp product was subsequently cut with ClaI and electrophoresed in a 1% agarose gel. Since R391 carries an additional ClaI recognition site in the corresponding amplified region, integration of R391 and SXT into $attR$ could be distinguished based on the sizes of the fragments obtained.

RESULTS

R391 integrates into $prfC$. Murphy and Pembroke (15) found that R391 integrates into the $E. coli$ chromosome in the region between 98.0 and 99.5 min. Since this area includes the SXT integration site, $prfC$, at 99.3 min (11), we speculated that R391 might also integrate into $prfC$ and encode a similar integration system. To test this possibility, we transferred R391 into $E. coli$ K-12 CAG18439, the strain we used to map the SXT integration site. With one of the resulting Kn^r Tc^r exconjugants, designated JO99, PCR was performed using primer pairs that were previously found to amplify the left and right junctions of SXT and the CAG18439 chromosome (primers 6 and 7 and primers 3 and 8 in Fig 1). Strikingly, the PCR products were identical in size to those derived using HW220 (CAG18439 $prfC$::SXT) DNA as the template (396 and 393 bp, respectively). Therefore, like SXT, R391 integrates into the 5′ end of $prfC$. The DNA sequences of the PCR products were determined and compared to the corresponding sequences of SXT-HW220 junction fragments published previously (11) (Fig. 1). This comparison revealed that the left ($attL$) and right ($attR$) attachment sites of R391 and SXT are virtually identical, differing only in 2 bp. Furthermore, like SXT, R391 restores the reading frame of $prfC$ (Fig. 1). Integrated into the chromosome, R391 is flanked by a 17-bp repeat sequence that probably corresponds to the core of the att site where strand exchange occurs during integration. The 17-bp R391 $attR$ differed from the SXT $attR$ by only 1 bp (a T at position 9 compared to a G in SXT$^+$ strains). The near identity of the R391 and SXT $attL$ and $attR$ sequences strongly suggests that the mechanism of chromosomal integration of R391 is very similar to that of SXT.

Identification of the R391 int gene. As part of an ongoing effort to determine the entire DNA sequence of SXT, we found that the DNA sequence of a region upstream of int_{SXT} had 95% identity to the 3′ end of $rumB$ from R391 (13). The $rumAB$ operon encodes proteins that are phylogenetically related to a superfamily of novel error-prone DNA polymerases (31). This finding was a further indication of the similarity of R391 and SXT and suggested that an R391 int gene might be located downstream of R391 $rumB$. We have taken advantage of the fact that during the cloning of the $rumAB$ operon, several recombinant plasmids were obtained that contained large fragments of extrachromosomal R391 DNA. Based on restriction analysis, it was determined that one of these plasmids, called pRLH421, contained approximately 7.5 kb 3′ to the $rumAB$ operon. The nucleotide sequence of this fragment was determined using standard sequencing protocols and revealed that 2.17 kbp downstream of the stop codon of $rumB$ there was a 1,242-bp open reading frame (ORF) which had 96% nucleotide sequence identity to int_{SXT}. The deduced amino acid sequence of this ORF was 99.5% identical to the integrase of SXT, with only three amino acids that differed (Y123F, S198G, and S333G). In both R391 and SXT, int is preceded by two nearly identical ORFs ($orf1$ and $orf2$ in Fig. 2A). Neither of these ORFs shows similarities to any known protein, and their functions remain to be determined.

Detection of an excised circular form of R391. int_{SXT} is required for the site-specific recombination between the $attL$ and $attR$ sequences of SXT, leading to the generation of an extra-
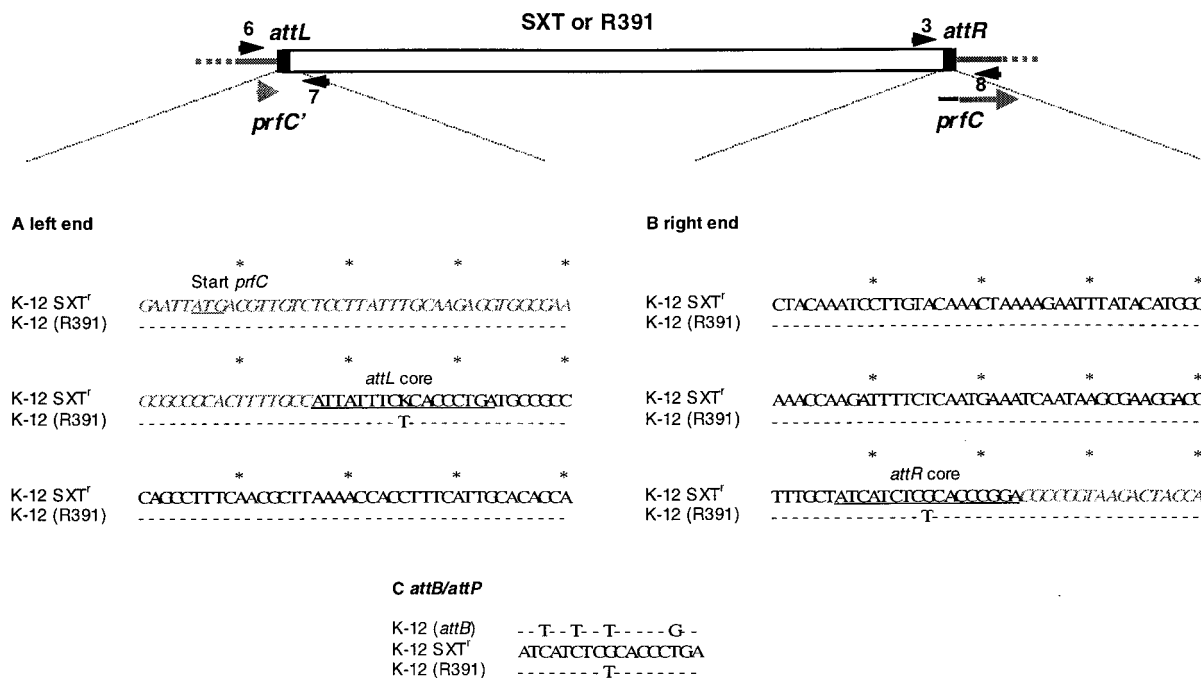
FIG. 1. Alignment of the nucleotide sequences of *attL* (A), *attR* (B), and *attP* (C) of SXT and R391. The primers (primers 6 and 7 and primers 3 and 8 [11]) used to amplify the junction fragments between the *E. coli* K-12 chromosome and R391 or SXT, respectively, are shown as black arrowheads. (A) Alignment of the sequences of *attL* of SXT (top) and R391 (bottom) obtained by sequencing the PCR product specific to the left junction fragment. Identical base pairs are represented by dashes. The 17-bp sequence encompassing the core of the *att* sites is underlined. The K in *attL* of SXT indicates that either T or G was found at this position (11). DNA specific to the chromosome of *E. coli* K-12 is in gray italic letters. (B) Alignment of the sequence of the right junction fragment of SXT (top) and R391 (bottom). Symbols are as in panel A. (C) Comparison of *attB* in the chromosome with *attP* of SXT and R391.

chromosomal circular form of SXT (11). The identification of an int_{R391} that is nearly identical to int_{SXT} suggested that R391 is also capable of excising from the chromosome and forming an extrachromosomal circle by a similar *int*-dependent mechanism. As no extrachromosomal DNA could be isolated by routine means from strains carrying R391, a more sensitive assay (11) based on PCR was used to identify an extrachromosomal circular form of R391. Since the primers in this assay are oriented toward the 5′ and 3′ ends of the integrated element, a product is only amplified if the element excises and circularizes (Fig. 2). Using this assay and control HW220 DNA (harboring SXT) as the template, the expected PCR product of 785 bp was obtained; with JO99 (harboring R391) as the template, the PCR amplification yielded a product of 2.5 kbp (Fig. 2). Thus, R391 also forms an extrachromosomal circle. However, the larger size of the PCR product from R391 than that from HW220 indicated that R391 possesses additional DNA at one of its ends. Indeed, sequence analysis of cloned extrachromosomal R391 DNA revealed that the R391 left end contains a region not present in SXT (Fig. 2). At 464 bp downstream of int_{R391}, a 1,212-bp ORF (*orf3*) was identified. The deduced amino acid sequence of *orf3* had 39% similarity and weak identity (~25%) to HipA of *E. coli* K-12 as well as 55% identity (73% similarity) to a reported HipA orthologue (y4DM) in the *Rhizobium* symbiosis plasmid pNGR234 (7). HipA, together with HipB, controls tolerance to certain peptidoglycan and DNA synthesis inhibitors (4). *orf3* seems to form an operon with *orf4*, since their likely start and stop codons are overlapping. Database comparisons with *orf4* revealed partial

identity to y4DL from plasmid pNGR234, the upstream gene of y4DM (7). Thus, this region appears to be arranged similarly in pNGR234 and R391, except that R391 seems to have lost about 100 amino acids in its y4DL homologue. Whether the gene products of *orf3* and *orf4* play a role in integration or transfer of R391 remains to be studied. The sequence of the core region of the R391 *attP* differed by only 1 nucleotide from the SXT *attP* sequence (Fig. 1C), corresponding to the position where *attR* varied (Fig. 1B). These minor variations in the R391 *attP* and *attR* compared with the corresponding SXT sequences are consistent with the previously proposed cut sites for the SXT integrase (11).

The extrachromosomal circular form of SXT is apparently a requisite intermediate in its self-transfer (11). We expected this to be the case for transfer of R391 as well. To obtain data to support this hypothesis, we constructed a derivative of JO100 (MG1655 Nal[r] containing R391) with a deletion in *int* (strain JO162). We were unable to detect the extrachromosomal circular form of R391 in this strain (Fig. 2); however, circle formation could be complemented in *trans* with *int* expressed from plasmid pINT391-19 (Fig. 2). JO162 was unable to transfer R391 to recipient cells, and pINT391-19 had to be present in both donor and recipient cells to restore transfer, as previously observed for SXT (11). This suggests that excision and circularization of R391 precede its self-transfer. Furthermore, these data suggest that in recipient cells, R391 is unable to stably replicate as an extrachromosomal circle and instead integrates into the recipient's chromosome to be maintained.
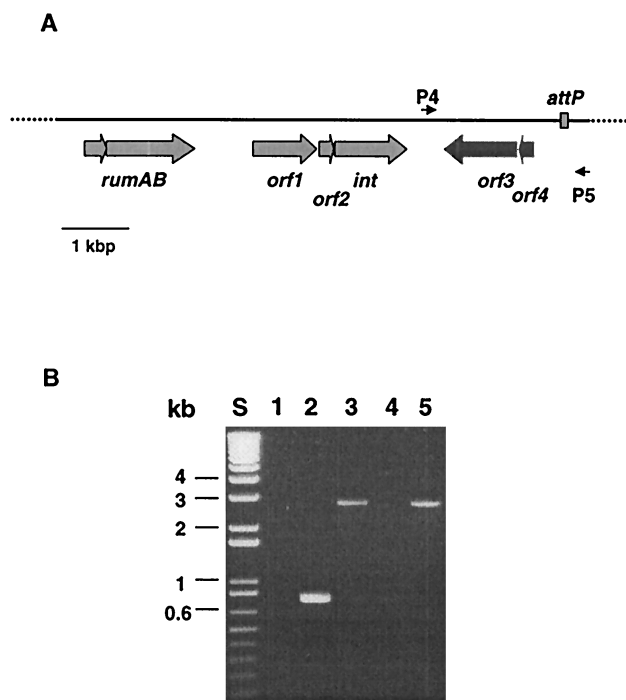
A

B

FIG. 2. Organization of the *int* region of R391 and detection of a circular extrachromosomal form of R391. (A) Organization of the region downstream of the *rumAB* operon in the circular form of R391. Following *rumAB*, five ORFs were identified, including the *int* gene of R391. *orf3* and *orf4* (black arrows) are not present in SXT. Also shown is *attP* and primers P4 and P5 used in the experiment in panel B. Note that these primers are oriented toward the chromosomal junctions of the integrated form of R391. (B) Ethidium bromide-stained 1% agarose gel of PCR products amplified using primers P4 and P5. Lanes: S, molecular size marker; 1, CAG18439; 2, BI537 (SXT⁺); 3, JO100 (R391); 4, JO162 (R391 *int⁻*); 5, JO162/pINT391-19.

R391 and SXT contain similar DNA sequences and have similar properties. Our ongoing study of the SXT DNA sequence has revealed that this element has a modular organization, with clusters of genes with related functions. We designed oligonucleotides to amplify different areas of SXT by PCR in order to test whether R391 has sequence similarity to SXT outside of the *int* region. Three PCR primer pairs that amplify internal fragments of three genes in the putative SXT *tra* cluster—*traC, traB,* and *traF* orthologues—were successfully used to amplify identically sized fragments from R391. Similarly, an ORF (*orfR*) at the "right" end of SXT with similarity to the *c*I repressor of phage 434 (17) was found in R391, as was an intergenic region 5′ of the *tra* cluster. Additional Southern hybridization analyses using probes specific to the *tra* genes (data not shown) suggested that the *tra* genes detected by PCR in R391 are not only closely related in sequence to those of SXT but are also similarly organized. Although R391 and SXT share many genes, these elements also encode different properties, such as resistance to antimicrobial agents and heavy metals. As predicted from these phenotypic differences, primers used for PCR amplification of genes that encode the resistance of SXT to sulfonamide, trimethoprim, chloramphenicol, and streptomycin did not yield products when JO99 DNA was used as the template. Conversely, we

detected *merC*, a gene of the mercury resistance operon of R391 (21), only in JO99 and not in HW220 (data not shown).

The similarity of R391 and SXT was not limited to DNA sequences and gene organization only; two defining properties of SXT were also found in R391. We previously found that transfer of SXT from donor cells is dependent on RecA (30). This was also the case for R391. When we introduced R391 into an *E. coli* MG1655 *recA56* mutant strain (KB1), the resulting exconjugant, JO139, transferred R391 with a frequency 3 to 4 orders of magnitude lower than that for the wild type. In contrast to our findings, Murphy and Pembroke (16) observed efficient transfer of R391 from donor cells carrying various *recA* alleles including the *recA56* allele used in our study. The reason for this difference is not known. The transfer frequency of SXT in liquid medium is greatly reduced compared to that on agar plates (30). We found a similar marked reduction in the transfer frequency of R391 in broth relative to plates (10^{-4} and 10^{-8}, respectively). This result is again in contrast to previously published observations (6). Different mating conditions or strain variations might account for these differences.

SXT and R391 coexist in tandem arrays in the chromosome. There have been relatively few studies concerning interactions between closely related constins like SXT and R391. Given the similarity of these two elements, including regions of DNA sequence identity, we explored whether they interfere with each other's transfer or maintenance. To assess this, we compared the transfer frequencies of R391 and SXT to a wild-type *E. coli* recipient strain with the transfer frequencies to isogenic strains harboring SXT or R391, respectively (Table 2). The presence of R391 in the recipient led to a minimal reduction in the measured transfer frequency of SXT from donor cells relative to recipients lacking R391 (Table 2). When SXT was present in the recipient cells, there was no detectable diminution in the R391 transfer frequency (Table 2). These data are consistent with previous observations (22, 32) suggesting that IncJ elements do not exclude each other.

PCR assays (Fig. 3) and Southern hybridizations (data not shown) were used to analyze the organization of R391 and SXT in the exconjugants that contained both elements. The PCR assays were designed to reveal the right and left junctions of these elements within the chromosome. For these assays, we took advantage of two DNA sequence differences that allow the ends of R391 and SXT to be distinguished. These are the

TABLE 2. The presence of SXT or R391 in recipient cells has a minimal effect on the transfer frequency of the other element

Donor	Relevant genotype	Recipient	Relevant genotype	Transfer frequency $(10^{-4})^a$ with selection^b for:		
				SXT	Kanamycin	SXT + kanamycin
BI537	SXT⁺	CAG18439		0.6	ND^c	ND
BI537	SXT⁺	JO99	R391⁺	0.1	ND	0.3
JO100	R391⁺	CAG18439		ND	1	ND
JO100	R391⁺	HW220	SXT⁺	ND	1	0.8

^{a} The frequency of exconjugant formation was calculated by dividing the number of exconjugant cells by the number of donor cells. Results are the means of three independent tests.
^{b} All exconjugants were selected on medium containing tetracycline and the indicated antibiotics.
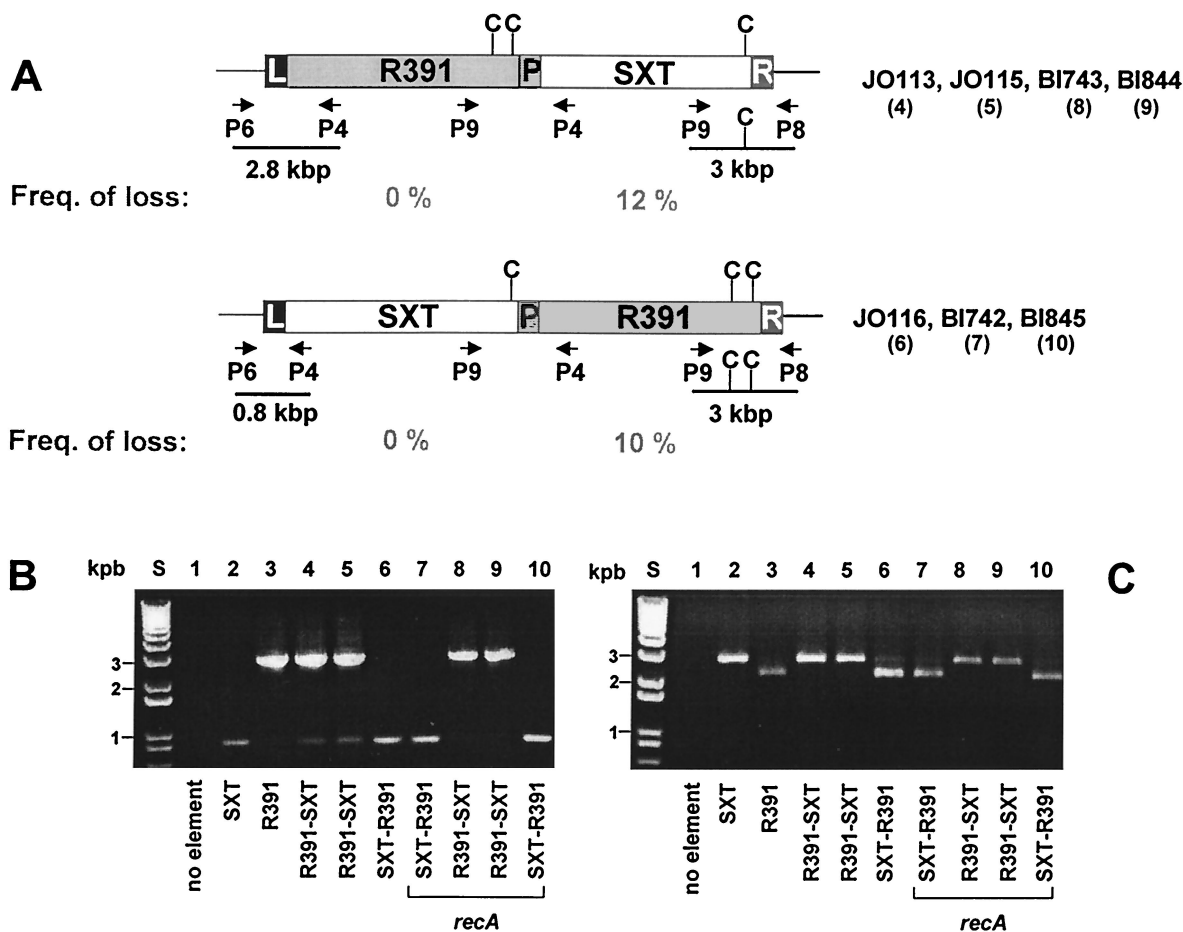^{c} ND, not done.

FIG. 3. Tandem arrangements of SXT and R391. (A) Schematic depiction (not to scale) of the tandem arrangements of R391 and SXT with either R391 (top) or SXT (bottom) present as the 5′ element. The primers (P4, P6, P8, and P9) and the predicted sizes of products resulting from PCR assays in both arrangements are also shown. The additional *Cla*I restriction site found at the right end of R391 is indicated. Also shown are the average frequencies of loss of SXT or R391, respectively, after ~20 generations of growth. Abbreviations: C, *Cla*I; L, *attL*; P, *attP*; R, *attR*. (B and C) Amplification of the left (B) and right (C) chromosome-element junction fragments. Ethidium bromide-stained 1% agarose gels of PCR products amplified using primers P4 and P6 (B) and P8 and P9 (C) are shown. PCR products in the experiment in panel C were digested with *Cla*I. Only the largest of these fragments is shown. The deduced order of R391 and SXT is indicated at the bottom, with the element flanked by *attL* as the first followed by the element flanked by *attR*. Lanes: S, molecular size marker; 1, CAG18439; 2, HW220; 3, JO99; 4, JO113; 5, JO115; 6, JO116; 7, BI742; 8, BI743; 9, BI844; 10, BI845.

additional DNA found at the left end of R391 (Fig. 2) and a *Cla*I site, which is present only at the right end of R391. In every exconjugant that contained both elements, amplification of the right and left element-chromosome junctions never revealed junctions characteristic of only one element (either R391 or SXT). Instead, in all cases analyzed, one junction was derived from SXT and the other from R391. This result suggests that the two elements are arranged in a tandem fashion in the chromosome in these cells (Fig. 3). In several strains containing both elements (e.g., JO113 and JO115 [lanes 4 and 5 in Fig. 3B]) we could detect both the R391 and SXT right junction fragment, with the latter junction appearing less abundant. We believe that the presence of the right SXT-chromosome junction in these cases reflects excision of R391. In RecA⁻ host cells containing both elements (strains BI742, BI743, BI844, and BI845 in Fig. 3), we also always observed junctions characteristic of both elements, suggesting that the process explaining the formation of Knʳ SXTʳ exconjugants does not involve homologous recombination between these

two related elements. In fact, we could use any of our RecA⁺ Knʳ SXTʳ exconjugants (e.g., JO113, JO115, or JO116) as donors of either SXTʳ, Knʳ, or SXTʳ and Knʳ in subsequent conjugation experiments. In these experiments, SXTʳ or Knʳ exconjugants had chromosomal junctions characteristic of SXT or R391, respectively, strongly suggesting that in the Knʳ SXTʳ donors the two elements remain as independent units and have not recombined.

Our results indicate that when either R391 or SXT integrates into a chromosome of a cell where the element's *attB* sequence is already occupied by the other element, the second element uses the resident element's *attL* or *attR* for integration rather than integrating into a secondary *att* site elsewhere on the chromosome. SXT and R391 exhibited different preferences for each other's *attL* and *attR* sequences. R391 preferentially integrated into the SXT *attL* (Table 3), whereas SXT integrated into the R391 *attL* or *attR* with similar frequencies. The mechanism underlying this differential specificity of *att* site selection is not clear. Apparently, the minor differences of

TABLE 3. Integration specificity of R391 and SXT[a]

Donor	Relevant genotype	Recipient	Relevant genotype	Frequency[b] of tandem arrangement (%)	
				R391-SXT	SXT-R391
BI537	SXT[+]	JO139	recA R391[+]	53 (20)	46 (18)
JO100	R391[+]	BI616	recA SXT[+]	91 (32)	9 (3)

[a] Either SXT or R391 was introduced into KB1 (recA) already harboring the other element. A PCR assay (using primers P6 and P4 [Fig. 3]) was used to determine where the second element integrated.

[b] The frequency of the two possible tandem arrangements is given as a percentage and in total numbers (in parentheses).

their integrases are not sufficient to explain this differential specificity, since expression of either int_{R391} or int_{SXT} in trans in a recipient cell did not confer this specificity to an incoming Int[−] element (data not shown).

Tandem arrangements of SXT and R391 were relatively stable in both RecA[+] and RecA^{−} cells. After ~20 generations of growth in LB broth without selection for either element, only ~10% of the SXT[r] Kn[r] exconjugants lost one of the two elements. Interestingly, there was a preferential loss of the element integrated into attR (Fig. 3). One potential explanation for this preference could be that the integrases of R391 and SXT bind to attR and attP (which is formed at the junction of the tandem elements) with greater affinity than to attL and attP. Subsequently, recombination between attP and attR results in excision and loss of the element located between these att sites.

Incompatibility between R391 and SXT. Previously, "incompatibility" between IncJ elements has been assessed in conjugation assays by measuring the frequency of loss of an unselected resident element on introduction of a second selected element (24). We adopted this approach to assess incompatibility between R391 and SXT. To our surprise, selection for R391 resulted in a higher frequency of loss of a resident SXT (26%) than the frequency of loss of a resident R391 (8%) when selection was applied for an incoming SXT (Table 4). Since we have never observed spontaneous loss of either R391 or SXT, these results reveal some type of incompatibility between these two elements. The magnitude of these frequencies are fairly similar to those previously reported as evidence of incompat-

ibility for IncJ elements (14, 24, 32). Nonreciprocal incompatibility has also been reported earlier for R997 and R391 (14).

The molecular bases of plasmid incompatibility are rooted in the factors that mediate either the replication or partitioning of these autonomously replicating genetic elements (19). Since constins like SXT and R391 are part of the chromosome, it seemed unlikely that these factors could mediate incompatibility between these elements. The considerable degree of homology between R391 and SXT suggested that homologous recombination between the two elements might explain the incompatibility we observed. However, this was clearly not the case; a similar frequency of loss of the unselected element occurred in recA recipients (Table 4). The inequality in the frequencies of loss of R391 and SXT suggests that some property that distinguishes the two elements plays a role in their incompatibility.

Since an integrase is required for integration and excision of these elements—key steps in either their gain or loss—we speculated that int may be important for the observed incompatibility. Consistent with this idea, an R391[+] int recipient had a lower frequency of loss of R391 than did an int[+] recipient in the incompatibility assay (Table 4). In contrast, an SXT[+] int recipient did not lose SXT at lower frequencies. A potential explanation for the disparate effects of the int deletions in the SXT and R391 int recipients is that int_{SXT} is less active than int_{R391}. In support of this idea, we found that deletion of int_{R391} from the donor strain JO162 resulted in a significant reduction in the frequency of loss of SXT from the recipient HW220 (Table 4). Furthermore, introduction of a plasmid containing int_{R391} into HW220 restored the higher frequency of loss of SXT from this strain, whereas introduction of the same vector containing int_{SXT} did not have this effect (Table 4). Thus, expression of int_{R391} in the recipient seems to facilitate the loss of one constin when the second is introduced. The effect of int_{R391} is most notable when it is expressed from an extrachromosomal element; it is less pronounced when int_{R391} is integrated within the chromosome (e.g., in JO100), perhaps because the chromosomal gene is expressed at lower levels. The imbalance between the loss of SXT and the loss of R391 may also reflect the fact that SXT is more frequently found on the right end of an array, the position from which elements are usually lost.

TABLE 4. Incompatibility between R391 and SXT

Donor	Relevant genotype	Recipient	Relevant genotype	% Loss[a] of:	
				SXT	R391
HW220	SXT[+]	JO100	R391[+]	NA[b]	8 (1–15)
BI537	SXT[+]	JO139	R391[+] recA	NA	4 (0–10)
HW220	SXT[+]	JO162	R391[+] int	NA	1.5 (0–3)
BI554/pINT6	SXT[+] int/int[+]	JO100	R391[+]	NA	11 (5–16)
JO100	R391[+]	HW220	SXT[+]	26 (7–52)	NA
JO100	R391[+]	BI616	SXT[+] recA	58 (39–77)	NA
JO100	R391[+]	BI554	SXT[+] int	39 (18–69)	NA
JO162/pINT6	R391[+] int/int[+]	HW220	SXT[+]	3 (0–8)	NA
JO162/pINT6	R391[+] int/int[+]	HW220/pINT391-19	SXT[+] int[+]/int[+]$_{R391}$	35 (12–58)	NA
JO162/pINT6	R391[+] int/int[+]	HW220/pINT18	SXT[+] int[+]/int[+]$_{SXT}$	4 (2–6)	NA

[a] A total of 100 exconjugants were scored for the resistance markers encoded on the resident, nonselected element, and the percent loss given represents the mean of three independent experiments. The range of the results of these tests is shown in parentheses.

[b] NA, not applicable.

DISCUSSION

We found that the conjugative, self-transmissible, integrating SXT element, originally derived from an Indian *V. cholerae* O139 isolate, is closely related to R391, a conjugative, self-transmissible, integrating "IncJ" element, originally derived from a South African *P. rettgeri* isolate. Both elements integrate into the same chromosomal attachment site (within the 5' end of *prfC*) in a *recA*-independent process that results from site-specific recombination between nearly identical element and chromosomal sequences. Chromosomal integration, as well as excision of these two elements, is dependent on their almost identical *int* genes. Both R391 and SXT can form extrachromosomal circular elements by recombination between their respective *attL* and *attR* sequences in an *int*-dependent manner. In addition to sharing DNA sequences and genes required for integration and excision, these elements contain related *tra* genes. Taken together, our data suggest that R391 and SXT are highly related constins with virtually identical mechanisms of integration into the chromosome and for dissemination. Despite their significant similarities, both elements also encode unique properties, such as resistance to specific antibiotics and heavy metals. These elements seem to consist of similar basic building blocks (modules encoding integration and transfer functions) to which have been added genes encoding unique features. It will be interesting to learn whether other previously defined IncJ elements share integration and transfer functions with SXT and R391. At least for R997 this seems likely, since this element is known to integrate between *uxuA* and *serB* on the *E. coli* chromosome (15), a region that includes *prfC*. The great similarity between SXT and R391, even though they are derived from different bacterial species and their highly disparate sites and dates of isolation, suggests that this type of constin may be considerably more pervasive than was previously suspected.

The interactions between related conjugative integrating elements have not been thoroughly investigated. Previous studies with the conjugative transposon Tn*916* and IncJ elements (18, 22, 32) have revealed that these elements do not prevent the transfer of a similar or identical element into a host already harboring such an element. Similar to this, our work showed that the presence of either R391 or SXT in a recipient cell had minimal to no detectable effect on the capability of the cell to serve as a recipient for the other element. This finding suggests that unlike plasmids such as F, these two elements do not encode surface exclusion mechanisms. Similarly, these elements do not seem to encode a repressor-mediated immunity function as described for bacteriophages such as λ or CTXφ (12, 25).

In cells carrying both R391 and SXT, the elements were arranged in tandem fashion on the chromosome. Homologous recombination played little or no role in the formation of these arrays. The arrays formed in *recA* recipients and either R391 or SXT could be transferred independently from a donor harboring both elements. Our data suggest that these tandem arrays were generated by site-specific recombination between an incoming element's *attP* sequence and the resident element's *attL* or *attR* sequence. The apparent lack of a role for homologous recombination as a form of interaction between R391 and SXT is in marked contrast to a recent report by Pembroke and Murphy, who studied the interaction between the two IncJ elements R997 and R391 (22). These authors concluded that homologous recombination is the predominant event which occurs following the introduction of R391 into a RecA⁺ host harboring R997 or vice versa. They concluded that in *recA* recipients the incoming element did not integrate but was maintained extrachromosomally as an autonomously replicating plasmid (22). This could be the case, although it is equally plausible that the extrachromosomal DNA they detected resulted from a shift in a equilibrium between the excised and integrated forms of R391 and R997 in cells harboring both of these IncJ elements. The differences between our observations and those of Pembroke and Murphy are most probably explained by the fact that we studied different IncJ elements. SXT and R391 may be less similar to each other than are R997 and R391. If this is the case, site-specific recombination mediated by the *int* genes of the elements may predominate over homologous recombination.

Incompatibility between R391 and SXT was determined by measuring the frequency of loss of an unselected resident element upon introduction of a second selected element, the established assay used to detect incompatibility of IncJ elements. While there was variability in our data regarding incompatibility between R391 and SXT (Table 4), there were always reproducible trends in these experiments that hint at a potential mechanism of incompatibility between R391 and SXT. Our data are consistent with a model where the level and/or specificity of activity of the donor and recipient integrases determines incompatibility. The factors regulating the amount or activity of these integrases are unknown; it is possible that there are differences in the production of integrase depending on whether an element is resident on the chromosome or present as an extrachromosomal circle. The three amino acid changes between the R391 and SXT integrases might also mediate subtle differences in the activities of these two enzymes, thereby explaining their different effects. A related factor that may play a role in the incompatibility of R391 and SXT is the apparently greater stability of the element in the 5'-most position in a tandem array. Since R391 had a preference for this site, this might in part account for the enhanced stability of this element.

Our findings demonstrate that the incompatibility between two constins that utilize the same *attB* is far less stringent than that observed for plasmids. Although the instability of R391 and SXT increased when both elements were present in a cell, these two elements can coexist relatively stably. Incompatibility between these constins is clearly a different process from that observed for plasmids. In fact, the term "incompatibility" has been previously applied only to replicating plasmids, and R391 and SXT in our assays do not appear to replicate as plasmids but ensure their vertical transmission by integrating into the chromosome. Assuming that our observations apply to other IncJ elements, the molecular bases of IncJ incompatibility are fundamentally different from the mechanisms known for other Inc groups. In this regard, it seems reasonable to abandon the term "IncJ incompatibility group" to describe this related group of constins and to apply a different term, perhaps "interference," to describe the interactions between similar IncJ elements.

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