CMLS, Cell. Mol. Life Sci. 59 (2002) 2065–2070
1420-682X/02/122065-06

CMLS Cellular and Molecular Life Sciences

Comparison of SXT and R391, two conjugative integrating elements: definition of a genetic backbone for the mobilization of resistance determinants

J. W. Beaber, V. Burrus, B. Hochhut and M. K. Waldor *

Division of Geographic Medicine/Infectious Diseases, New England Medical Center and Tufts University School of Medicine and Howard Hughes Medical Institute, NEMC 041, 750 Washington St., Boston, Massachusetts 02111 (USA), Fax: +1 617 636 5292, e-mail: mwaldor@lifespan.org

Abstract. The SXT element (SXT) is becoming an increasingly prevalent vector for the dissemination of antibiotic resistances in *Vibrio cholerae*. SXT is a member of a larger family of elements, formerly defined as IncJ plasmids, that are self-transmissible by conjugation and integrate site-specifically into the host chromosome. Comparison of the DNA sequences of SXT and R391, an IncJ element from *Providencia rettgeri*, indicate that these elements consist of a conserved backbone that mediates the regulation, excision/integration and conjugative transfer of the elements. Both elements have insertions into this backbone that either confer the element-specific properties or are of unknown function. Interestingly, the conserved SXT and R391 backbone apparently contains hotspots for insertion of additional DNA sequences. This backbone represents a scaffold for the mobilization of genetic material between a wide range of Gram-negative bacteria, allowing for rapid adaptation to changing environments.

Key words. SXT; conjugative transposon; R391; gene transfer; *Vibrio cholerae*; comparative genomics.

Discovery of SXT and related elements in *Vibrio cholerae*

In late 1992, *Vibrio cholerae* O139 emerged in India and Bangladesh as the first non-O1 serogroup of *V. cholerae* to cause epidemic cholera. Microbiological and molecular characterization of *V. cholerae* O139 revealed that this newly emerged serogroup was closely related to the El Tor biotype of *V. cholerae* O1 that it initially replaced as the predominant cause of cholera on the Indian subcontinent [1]. Besides the novel serogroup antigen, *V. cholerae* O139 isolates also differed from El Tor O1 isolates in their antibiotic resistance profiles. Unlike the El Tor strains, O139 strains were resistant to sulfamethoxazole, trimethoprim, chloramphenicol and streptomycin. Initial characterization of the genes encoding these resistances indicated that they resided on a novel mobile genetic element designated SXT (for sulfa and trimethoprim) [2]. Although these antibiotic resistance genes were transmissible between *V. cholerae* strains by conjugation, plasmids were not detected in *V. cholerae* O139 isolates [2]. Instead, analyses of genomic DNA derived from transconjugant cells by pulsed field gel electrophoresis revealed that these antibiotic resistance determinants were integrated into a single site in the chromosome. Additionally, these transconjugants were able to serve as donors, indicating that SXT was a conjugative, self-transmissible integrating element similar to conjugative transposons [2]. The host range of SXT was not limited to *V. cholerae*, as the element could be transferred to several Gram-negative bacteria.

When El Tor O1 *V. cholerae* reemerged in India, these strains, unlike their predecessors, were resistant to the same antibiotics as the O139 strains that they replaced [3]. The resistance determinants in these strains were also found to be located on a self-transmissible element closely related but not identical to SXT [2, 4]. Although more recent O139 isolates from India are no longer resistant to sulfamethoxazole or trimethoprim [5], molecular analyses indicate that these isolates still harbor an SXT-related element [6]. In addition, a recent study examining *V.*

* Corresponding author.

cholerae clinical isolates from Mozambique and South Africa [7] suggests that SXT-like elements are now widespread in Africa as well as Asia. Finally, an SXT-related element has recently been detected in *Providencia alcalifaciens* clinical isolates from Bangladesh, indicating that the SXT group of conjugative integrating elements is found outside of *V. cholerae* [6].

SXT is related to IncJ elements

SXT appears to be a member of a larger family of mobile elements [8], once thought to be plasmids of the IncJ group, all mediating resistance to antibiotics and/or metals. Except under unusual laboratory conditions [9, 10], extrachromosomal DNA has not been isolated from any IncJ element [11], suggesting that like SXT, they are conjugative transposons, integrating into their hosts' chromosomes [12]. These elements have been found in a variety of pathogenic γ proteobacteria. R391, the first described IncJ element, was initially isolated from a *Providencia rettgeri* clinical isolate in South Africa in 1972 [13] and encodes resistance to mercury and kanamycin. Other reported IncJ elements include pJY1, isolated from *Vibrio* spp. in the Philippines [14], R997, isolated from *Proteus mirabilis* in India [15], and pMERPH, isolated from *Shewanella putrefaciens* in the UK [16]. Although only two of these elements, SXT and R391, have been examined in detail [2, 12], preliminary phenotypic analyses suggest that the other elements are closely related. SXT and the IncJ elements are gaining recognition as widespread mobile elements that can disseminate antibiotic resistances and probably other important properties among bacterial populations.

Properties of SXT and R391

SXT was found to be integrated near the 5′ end of the *prfC* gene in *V. cholerae* [4]. The integrated element appears to be very stable, as loss of SXT has never been observed, even after growth without selection for many generations [4]. Following transfer to a new host, SXT integrates into the same site in the *V. cholerae* and *Escherichia coli* chromosomes [2, 4]. SXT site-specific integration into and its excision from the chromosome require an element-encoded integrase, Int, that bears similarity to phage-encoded tyrosine recombinases. SXT chromosomal integration and excision are similar to the chromosomal integration and excision described for lambdoid phages. These similarities include (i) formation of a circular extrachromosomal intermediate through recombination of sequences at the left and right ends (*attL* and *attR*, respectively) of the integrated element, (ii) recombination between relatively short element (*attP*) and chromosomal (*attB*) sequences in a *recA*-independent fashion and (iii) the requirement of a tyrosine recombinase to mediate this recombination [4]. SXT integration disrupts the 5′ end of *prfC*, a nonessential gene encoding RF3, a protein involved in the termination of translation. The 3′ end of SXT encodes a novel 5′ coding sequence for *prfC* and a promoter that leads to expression of functional RF3. SXT excision from the chromosome restores the wild-type copy of *prfC* [4]. This disruption/restoration phenomenon has also been observed in integration of the Gifsy-1 phage integration into *lepA* [17], and a cryptic integrating element derived from *Mesorhizobium loti* into a transfer RNA (tRNA) gene [18]. R391 was found to be integrated between 98.0 and 99.5 minutes on the *E. coli* chromosome [19]. Subsequently, Hochhut et al. localized the insertion site to *prfC* and identified an R391-encoded integrase nearly identical to that of SXT, indicating that the mechanisms for R391 integration and excision are virtually identical to those of SXT [8].

Although an extrachromosomal circular form of SXT has been observed and is thought to be the transfer intermediate, an autonomously replicating circular form of SXT has not been identified [2, 4]. Consistent with this observation, analysis of the SXT DNA sequences did not reveal any genes related to known replication factors [20]. Also, transconjugant formation requires *int* expression in recipient cells, suggesting that the circular extrachromosomal form of the element cannot be stably maintained without its integration [4]. Instead, SXT maintenance apparently requires its integration into the chromosome. Under certain experimental conditions an extrachromosomal, circular form of R391 and R997 has successfully been isolated [9, 10]. When the investigators transferred R391 into a *recA*-deficient strain containing R997, both elements were able to coexist, and an extrachromosomal element corresponding to the approximate size of R391 was isolated. The same was true for R997 in the converse experiment [10]. It remains to be determined whether these observations reflect detection of an autonomous replicative form of these elements or a shift in equilibrium between the excised and integrated forms of R391 and R997 in cells containing both elements.

DNA sequence analysis indicates that the conjugative apparatus utilized by SXT and R391 is related to that of the F plasmid [20, 21]. Following transfer of R391 and R997 to 'bald' strains of *E. coli,* long, flexible pili were observed [22]. The transfer frequency of both R391 and SXT are relatively low (10^{-4}–10^{-5} exconjugants per donor in *E. coli*, and even lower in *V. cholerae*) [2, 12, 13], while R997 transfers at a higher frequency (10^{-3} per recipient) [22]. While both SXT and R391 are able to transfer on solid media, only R391 is able to transfer at a relatively high frequency in broth [13]. Also, the elements differ in their requirement for *recA* in conjugative transfer. The frequency of SXT transfer drops dramatically in the absence

of RecA in the donor [2], while R391 transfer is unaffected [12]. The basis for this difference in not understood. SXT and R391 have also been shown to mobilize chromosomal DNA in a manner similar to an Hfr [11, 23]. SXT is able to mobilize certain plasmids in trans as well [23].

Although SXT and the IncJ elements are not plasmids and do not appear to encode partitioning or replication genes, they do exhibit a form of incompatibility [10, 24, 25]. IncJ 'incompatibility' has been assessed by measuring the frequency of loss of an unselected resident element in a recipient upon introduction of a second element. Two recent studies observed this type of incompatibility between R997 and R391 [10], and between R391 and SXT [8]. The molecular mechanisms for this type of incompatibility have yet to be determined, but surface exclusion does not appear to play a role. When R391 was introduced into a cell containing SXT, or vice versa, and both elements were selected for, both elements were stably maintained in the cell in tandem arrays [8]. It was proposed that the incoming element recognizes *attL* or *attR* as a target sequence for integration.

Genomic Comparison Between SXT and R391

The complete nucleotide sequences of both SXT (99.5 kb) and R391 (89 kb) have been determined [20, 21]. Comparison of these sequences indicates that the elements are very closely related. In fact, the elements share approximately 65 kb of DNA exhibiting greater than 95% identity at the nucleotide level (fig. 1). Our recent work [20] suggests that this conserved DNA (shown in green in fig. 1) includes the genes for conjugative transfer, excision/integration and regulation of transfer, and therefore seems to constitute a minimal 'backbone' of genetic information required for the mobility of this family of elements. While all of the SXT genes found to be essential for conjugative transfer are conserved in R391, not all of the conserved sequences between the two elements were required for conjugative transfer. For example, deletion of the region from *s024* to *s040*, which is largely conserved between the two elements, had no detectable effect on the excision or transfer of SXT [20]. Given their conservation, these gene products may confer a selective advantage that remains to be determined. Thus, the minimal conjugative element is smaller than the conserved backbone.

The backbone contains three modules, or clusters of genes of related function, required for the conjugative transfer and maintenance of these elements: an integration module encoding the functions involved in integration and excision, a conjugation module encoding proteins forming the mating apparatus and for processing transferred DNA as well as containing an origin of transfer (*oriT*), and a regulation module controlling the expression of the other two modules and, presumably, coordinating the events leading to transfer. The integration module consists of the attachment site (*attP*) and the operon from *s003* to *int*. R391 has two open reading frames (ORFs) upstream of *s003* (indicated in red, fig. 1); the DNA in this region is largely conserved within SXT, but the ORFs were not annotated due either to frame shifts or sequencing errors. Unlike SXT, R391 contains three genes of unknown function downstream of *int*; this separation of the *int* gene from the attachment site is unusual.

The conjugation modules are absolutely conserved in both gene content and gene order. This module is related to the conjugation modules found in the conjugative plasmids R27 from *Salmonella serovar Typhi* [26], pNL1 from *Sphingomonas aromativicans* [27] and Rts1 from *E. coli* [28], and the Gonococcal Genetic Island (GGI) from the chromosome of *Neisseria gonorrhea* [J. P. Dilliard, personal communication], though the genetic arrangements differ. These genes are divided into four separate groups in both elements, group 1 (*traI* to *s043*), group 2 (*traL* to *traA*), group 3 (*s054* to *traN*) and group 4 (*traF* to *traG*), although the DNA sequences dividing these groups are not conserved. With the exception of the genes between group 2 and group 3 in SXT, each transfer group is separated by divergently oriented genes, suggesting independent regulation.

The regulation module of SXT consists of the transcriptional activators, SetC and SetD, and the putative repressor, SetR [20]. SetC and SetD are similar to FlhC and FlhD, the master regulators of flagellar transcription, and SetR is similar to the repressor CI from phage λ. The putative R391 SetC, SetD and SetR gene products are 95, 100 and 100% identical, respectively, to their SXT counterparts. Given this similarity, we presume that these elements share common regulatory mechanisms.

In addition to the three modules required for mobilization and maintenance of SXT, both elements contain two other conserved regions, *s024* to *s040* (see above) and *s063* to *s073*, that are not essential for SXT transfer. The later region is 12.1 kb in length and found between transfer groups 3 and 4. This region contains an ORF (*ssb*) that encodes a protein that has homology to single-stranded DNA-binding proteins, a common feature in conjugative plasmids [29], and another ORF (*s065*) whose product is similar to a phage recombinase. The rest of the gene products in this region do not have significant similarity to anything in GenBank. While deletion of this region resulted in a 1000× reduction in transfer frequency [20], smaller deletions indicate that no single gene accounts for this defect.

Genes are inserted in several locations in the conserved backbone (shown in blue for SXT and in yellow for R391, fig. 1) that in some cases confer the element-specific properties. Some of these insertions appear to have been mediated by transposons, including a composite transposon-like element carrying SXT's resistance de-

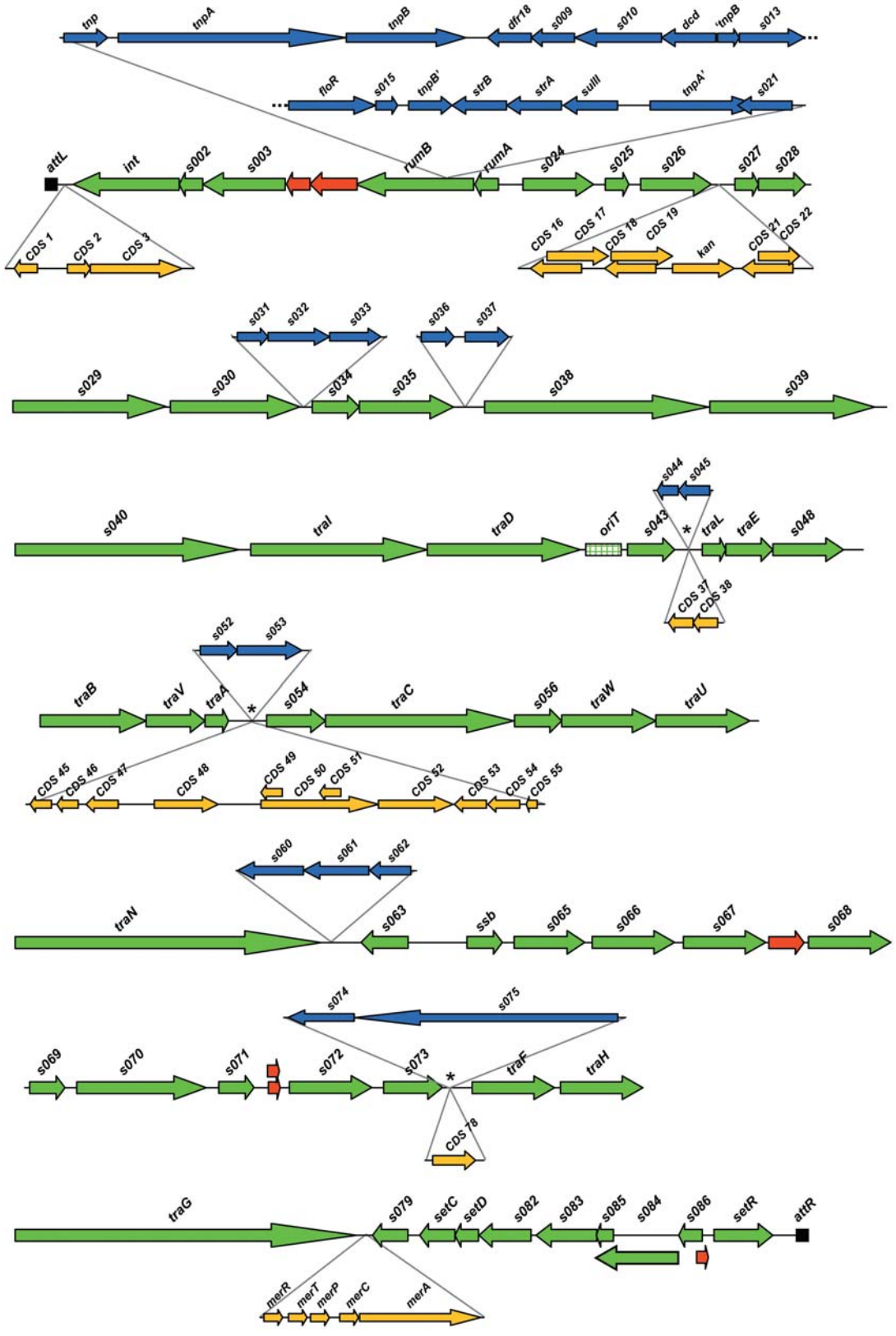

Figure 1. Comparison of the SXT and R391 DNA sequences. Shared DNA sequences are indicated by the green arrows. SXT gene designations are used to annotate shared genes. Red arrows indicate genes annotated in the R391 sequence but not in SXT, even though the sequences are generally conserved. SXT-specific DNA is indicated by the blue arrows, R391-specific DNA by the yellow arrows. Gray lines indicate the point of insertion for the element-specific DNA. The location of sequences depicted in figure 2 is indicated by asterisks. The origin of transfer is indicated by the hatched box.

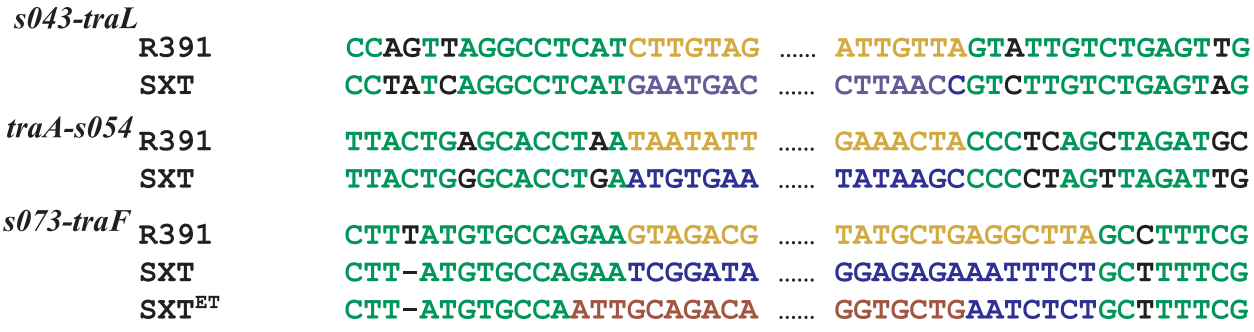

Figure 2. ClustalW alignment of sequences flanking the three loci containing different *tra* region inserts in SXT, R391 and, in the case of *s073-traF*, SXT^{ET}. Backbone DNA is depicted in green, SXT-specific DNA in blue, R391-specific DNA in yellow and SXT^{ET}-specific DNA in red. Nucleotide differences in backbone DNA are indicated in black. Alignments were performed with AlignX software (Informax, Bethesda, MD).

terminants inserted into *rumB* and a putative transposon carrying kanamycin resistance in R391 [6]. In other cases, analysis of the inserted sequences does not suggest a mechanism of acquisition. These include the mercury resistance genes in R391 and a variety of different inserts that confer no known phenotype in both elements. While we hypothesize that these genetic differences reflect insertions into the backbone, an equally plausible explanation is that some differences reflect deletions from a progenitor element.

Interestingly, three sites in the backbone element appear to be hotspots for the introduction of novel DNA. Both R391 and SXT contain different inserts into their respective conjugation modules, between *s043* and *traL*, *traA* and *s054*, and *s073* and *traF* (fig. 1). These insertions potentially divide the transfer region into four independently regulated transcriptional units. Remarkably, the insertions appear to have occurred at the same nucleotide in both elements at all three loci (fig. 2). There are no obvious insertion sequences or repeats flanking these inserts, and only the insert between *traA* and *s054* in R391 contains a putative transposase, *cds48*. A recent study examining the antibiotic resistance determinants in other SXT-like elements noted a third, different insertion between *s073* and *traF* in SXT^{ET}, an SXT-like element derived from a *V. cholerae* El Tor O1 clinical isolate [6]. The point of insertion for this cassette is within a few base pairs of the common insertion site in SXT and R391 (fig. 2). The insert contains a novel type of integron that constitutes a fourth class of resistance integrons. It contains a novel integrase and five integron cassettes, each with a characteristic *attC* [6]. The integrase presumably explains the presence of the five cassettes, but does not explain the presence of the integron in the element. The mechanism(s) of acquisition of these insertions are unknown.

Conclusions

SXT and R391 share a conserved backbone that has acquired additional DNA conferring element specific properties. Both contain three functional modules for element integration, conjugation and regulation essential for their dissemination. These shared modules, however, only represent a portion of the conserved genetic content making up the backbone of the two elements. For the most part, the remainder of this backbone consists of genes of unknown function that may confer some fitness advantage. Both elements contain inserts into this scaffold that impart their element-specific properties, namely resistances to antibiotics and heavy metals. Other element-specific inserts are of unknown function; three of these insertions revealed that the backbone apparently contains hotspots for recombination. The genetic differences between these two elements likely reflect the distinct evolutionary pressures encountered by their hosts. The backbone of SXT and R391 represents a dynamic scaffold facilitating the flow of genetic information among host bacteria, thereby aiding in their survival and spread.

1 Cholera Working Group (1993) Large epidemic of cholera-like disease in Bangladesh caused by *Vibrio cholerae* O139 synonym Bengal. International Centre for Diarrhoeal Diseases Research, Bangladesh. Lancet **342:** 387–390
2 Waldor M. K., Tschape H. and Mekalanos J. J. (1996) A new type of conjugative transposon encodes resistance to sulfamethoxazole, trimethoprim and streptomycin in *Vibrio cholerae* O139. J. Bacteriol. **178:** 4157–4165
3 Yamamoto T., Nair G. B., Alpert M. J., Parodi C. and Takeda Y. (1994) In vitro susceptibilities of *Vibrio cholerae* O1 and O139 to antimicrobial agents and appearance of drug resistance plasmids in *Vibrio cholerae* O139. Presented at the 30th Joint Conference U.S. – Japan Cooperative Medical Science Program Cholera and Related Diarrheal Diseases Panel, Fukuoka, Japan
4 Hochhut B. and Waldor M. K. (1999) Site-specific integration of the conjugal *Vibrio cholerae* SXT element into *prfC*. Mol. Microbiol. **32:** 99–110

5 Mukhopadhyay A. K., Basu A., Garg P., Bag P. K., Ghosh A., Bhattacharya S. K. et al. (1998) Molecular epidemiology of reemergent *Vibrio cholerae* O139 Bengal in India. J. Clin. Microbiol. **36:** 2149–2152
6 Hochhut B., Lotfi Y., Mazel D., Faruque S. M., Woodgate R. and Waldor M. K. (2001) Molecular analysis of the antibiotic resistance gene clusters in the *Vibrio cholerae* O139 and O1 SXT constins. Antimicrob. Agents Chemother. **45:** 2991–3000
7 Dalsgaard A., Forslund A., Sandvang D., Arntzen L. and Keddy K. (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
8 Hochhut B., Beaber J. W., Woodgate R. and Waldor M. K. (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
9 Ho C., Kulaeva O. I., Levine A. S. and Woodgate R. (1993) A rapid method for cloning mutagenic DNA repair genes: isolation of *umu*-complementing genes from multidrug resistance plasmids R391, R446b and R471a. J. Bacteriol. **175:** 5411–5419
10 Pembroke J. T. and Murphy D. B. (2000) Isolation and analysis of a circular form of the IncJ conjugative transposon-like elements, R391 and R997**:** implications for IncJ incompatibility. FEMS Microbiol. Lett. **187:** 133–138
11 Nugent M. (1981) A conjugative 'plasmid' lacking autonomous replication. J. Gen. Microbiol. **126:** 305–310
12 Murphy D. B. and Pembroke J. T. (1995) Transfer of the IncJ plasmid R391 to recombination deficient *Escherichia coli* K12: evidence that R391 behaves as a conjugal transposon. FEMS Microbiol. Lett. **134:** 153–158
13 Coetzee J. N., Datta N. and Hedges R. W. (1972) R factors from *Proteus rettgeri*. J. Gen. Micro. **72:** 543–552
14 Yokota T. and Kuwahari S. (1977) Temperature sensitive R plasmid obtained from naturally isolated drug-resistant *Vibrio cholera* (biotype El Tor). Antimicrob. Agents Chemother. **11:** 13–20
15 Matthews M., Hedges R. W. and Smith J. T. (1979) Types of β-lactamase determined by plasmids in Gram-negative bacteria. J. Bacteriol. **138:** 657–704
16 Hedges R. W. (1974) R factors from Providence. J. Gen. Microbiol. **181:** 171–181
17 Figueroa-Bossi N. and Bossi L. (1999) Inducible prophages contribute to *Salmonella* virulence in mice. Mol. Microbiol. **33:** 167–176
18 Zhao S. and Williams K. P. (2002) Integrative genetic element that reverses the usual target gene orientation. J. Bacteriol. **184:** 859–860
19 Murphy D. B. and Pembroke J. T. (1999) Monitoring of chromosomal insertions of the IncJ elements R391 and R997 in *Escherichia coli* K-12. FEMS Microbiol. Lett. **174:** 355–361
20 Beaber J. W., Hochhut B. and Waldor, M. K. (2002) Genomic and functional analyses of SXT an integrating antibiotic resistance gene transfer element derived from *Vibrio cholerae*. J. Bacteriol. **184:** 4259–4269
21 Boeltner D., MacMahon C., Pembroke J. T., Strike P. and Osborn A. M. (2002) R391: a conjugative integrating mosaic comprised of phage, plasmid and transposon elements. J. Bacteriol. **184:** 5158–5169
22 Bradley D. E. (1980) Determination of pili by conjugative bacterial drug resistance plasmids of incompatibility groups B, C, H, J, K, M, V and X. J. Bacteriol. **141:** 828–837
23 Hochhut B., Marrero J. and Waldor M. K. (2000) Mobilization of plasmids and chromosomal DNA mediated by the SXT element, a constin found in *Vibrio cholerae* O139. J. Bacteriol. **182:** 2043–2047
24 Hedges R. W., Jacob A. E., Datta N. and Coetzee J. N. (1975) Properties of plasmids produced by recombination between factors of group J and FII. Mol. Gen. Genet. **140:** 289–302
25 Peters S. E., Hobman J. L., Strike P. and Ritchie, D. A. (1991) Novel mercury resistance determinants carried by IncJ plasmids pMERPH and R391. Mol. Gen. Genet. **228:** 294–299
26 Sherburne C. K., Lawley T. D., Gilmour M. W., Blattner F. R., Burland V., Grotbeck E. et al. (2000) The complete DNA sequence and analysis of R27, a large IncHI plasmid from *Salmonella typhi* that is temperature sensitive for transfer. Nucleic Acids Res. **28:** 2177–2186
27 Romine M. F., Stillwell L. C., Wong K. K., Thurston S. J., Sisk E. C., Sensen C. et al. (1999) Complete sequence of a 184-kilobase catabolic plasmid from *Sphingomonas aromaticivorans* F199. J. Bacteriol. **181:** 1585–1602
28 Murata T., Ohnishi M., Ara T., Kaneko J., Han C. G., Li Y. F. et al. (2002) Complete nucleotide sequence of plasmid Rts1: implications for evolution of large plasmid genomes. J. Bacteriol. **184:** 3194–3202
29 Ruvolo P. P., Keating K. M., Williams K. R. and Chase J. W. (1991) Single-stranded DNA binding proteins (SSBs) from prokaryotic transmissible plasmids. Proteins **9:** 120–134