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Molecular Analysis of Antibiotic Resistance Gene Clusters in *Vibrio cholerae* O139 and O1 SXT Constins

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Many recent Asian clinical *Vibrio cholerae* E1 Tor O1 and O139 isolates are resistant to the antibiotics sulfamethoxazole (Su), trimethoprim (Tm), chloramphenicol (Cm), and streptomycin (Sm). The corresponding resistance genes are located on large conjugative elements (SXT constins) that are integrated into *prfC* on the *V. cholerae* chromosome. We determined the DNA sequences of the antibiotic resistance genes in the SXT constin in MO10, an O139 isolate. In SXT^{MO10}, these genes are clustered within a composite transposon-like structure found near the element's 5′ end. The genes conferring resistance to Cm (*floR*), Su (*sul*II), and Sm (*strA* and *strB*) correspond to previously described genes, whereas the gene conferring resistance to Tm, designated *dfr18*, is novel. In some other O139 isolates the antibiotic resistance gene cluster was found to be deleted from the SXT-related constin. The El Tor O1 SXT constin, SXT^{ET}, does not contain the same resistance genes as SXT^{MO10}. In this constin, the Tm resistance determinant was located nearly 70 kbp away from the other resistance genes and found in a novel type of integron that constitutes a fourth class of resistance integrons. These studies indicate that there is considerable flux in the antibiotic resistance genes found in the SXT family of constins and point to a model for the evolution of these related mobile elements.

The intercellular spread of the genetic determinants of resistance to antimicrobial agents is facilitated by mobile genetic elements, such as conjugative plasmids and conjugative transposons. The antibiotic resistance genes in these elements are often located within transposons and/or integrons, elements that facilitate the intracellular movement of genes. Two types of transposons have been found to contain resistance genes. Class I transposons, also known as composite transposons, consist of two insertion sequence (IS) elements that flank additional DNA sequences, such as resistance genes. Class II transposons do not contain recognizable IS elements; instead, the genetic information for their transposition and other phenotypes (including antibiotic resistances) is bordered by 35- to 110-bp inverted repeats (reviewed in reference 10). Integrons also play a major role in the spread of antibiotic resistance genes in gram-negative bacteria (32). Integrons are gene-capturing systems that incorporate gene cassettes and convert them to functional genes (31, 32). Integrons characteristically encode an integrase (*intI*) that mediates recombination between a sequence in the gene cassette (*attC*) and an integron-associated sequence (*attI*). This results in integration of the cassette downstream of a resident promoter to permit expression of the encoded protein. While integrons often are found in plasmids and usually contain antibiotic resistance genes, they can also be located on the chromosome and can contain genes that do not specify resistance to antibiotics (4, 26). To date, three classes of resistance integrons have been described based on similarities in the integrase sequences. Class I integrons usually contain the gene *sulI*, encoding sulfamethoxazole resistance, at their 3′ end (32). Recently, a new type of integron, collectively called chromosomal superintegrons, has been found in the chromosomes of several species belonging to the gamma proteobacteria, including *Vibrio cholerae* (18, 26, 34).

V. cholerae is the causative agent of the severe and sometimes lethal diarrheal disease cholera. While the genetic bases of resistance to antibiotics in *V. cholerae* have not been extensively characterized, antibiotic resistance determinants are usually found on plasmids in this organism (13, 17, 40). Historically, only the O1 serogroup of *V. cholerae* has been associated with epidemic cholera. However, in late 1992 in India and Bangladesh, a novel serogroup designated *V. cholerae* O139 emerged and gave rise to major cholera outbreaks. Initially, *V. cholerae* O139 replaced *V. cholerae* El Tor O1 as the predominant cause of cholera on the Indian subcontinent (5). Microbiologic and genetic characterization of *V. cholerae* O139 revealed that this serogroup arose from *V. cholerae* O1 El Tor by horizontal gene transfer and substitution of the genes encoding the O139 serogroup antigen for the genes encoding the O1 serogroup antigen (3, 9, 38, 42). Besides the novel serogroup antigen, the initial O139 isolates could be distinguished from the O1 strains they replaced by characteristic resistances to the antibiotics sulfomethoxazole (Su), trimethoprim (Tm), chloramphenicol (Cm), and low levels of streptomycin (Sm). In MO10, a 1992 clinical O139 isolate, the genes encoding these

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resistances were found to be located on a novel transmissible genetic element designated the SXT element (referred to here as SXT^{MO10}) (44).

Though it is self-transmissible, an autonomously replicating extrachromosomal form of SXT^{MO10} has not been isolated; instead, this ~100-kbp element is always integrated into the 5′ end of the chromosomal gene *prfC*. SXT^{MO10} encodes an integrase related to the λ family of site-specific recombinases, and we have shown that the integrase mediates the element's integration and its chromosomal excision, which generates a circular episome (21). This circular but apparently nonreplicating form of the element is believed to be a requisite intermediate for its conjugative transfer between *V. cholerae* strains, as well as between other gram-negative bacteria. We proposed a new term, constin, an acronym for the element's properties (conjugative, self-transmissible, and integrating) to describe SXT^{MO10} and other elements with similar features.

After the extensive cholera outbreaks caused by *V. cholerae* O139 strains, El Tor O1 *V. cholerae* strains reemerged in 1994 as the predominant cause of cholera on the Indian subcontinent. In contrast to the El Tor O1 strains before the O139 outbreak, these reemerged El Tor strains, like the initial O139 isolates, were resistant to Su, Tm, Cm, and Sm (48). The corresponding resistance genes were found to be located in a constin (designated here SXT^{ET}) that is closely related but not identical to SXT^{MO10} (21, 44). Variation is also evident in more recent O139 isolates from India, as these are generally no longer resistant to Su and Tm (28). However, molecular analyses have revealed the presence of an SXT^{MO10}-like element integrated into *prfC* in these strains, indicating that they still harbor constins related to SXT^{MO10} (21).

SXT-like elements are not unique to *V. cholerae* O139. For example, the IncJ element R391 that mediates kanamycin (Kn) and mercury resistance, originally derived from a South African *Providencia retgerii* isolate (8), is functionally and genetically related to SXT^{MO10} (20). Analysis of these two elements suggested that they consist of similar basic building blocks—modules encoding integration and transfer functions—to which have been added genes encoding defining features, such as antibiotic resistance genes (20).

In this study, we determined the sequence and organization of the antibiotic resistance genes in SXT^{MO10} and compared them to those of other SXT constins. The SXT^{MO10} resistance genes are embedded in a ~17.2-kbp composite transposon-like element that interrupts the SXT-encoded *rumAB* operon. A deletion event, likely mediated by recombination between duplicated sequences in this region, accounts for the Su and Tm sensitivity of recent O139 isolates. In SXT^{ET}, unlike in SXT^{MO10}, resistance to Tm is encoded outside the cluster of resistance genes; instead, the Tm resistance determinant is found in a novel class of integrons located far away from the remainder of the antibiotic resistance genes within SXT^{ET}. By comparison, the Kn resistance gene in R391 is found to be part of a transposon containing IS*26* elements that is located ~3 kbp 5′ to the R391 *rumAB* operon. Overall, these studies indicate that the antibiotic resistance determinants on constins are often part of dynamic genetic structures that allow relatively rapid alteration of the properties encoded by a constin.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains used in this study are described in Table 1. Bacterial strains were routinely grown in Luria-Bertani (LB) broth (2) at 37°C and stored at −70°C in LB broth containing 20% (vol/vol) glycerol. Antibiotics were used at the following concentrations: ampicillin (Ap), 100 mg $liter^{-1}$; Kn, 50 mg $liter^{-1}$; Su, 160 mg $liter^{-1}$; Tm, 32 or 250 mg $liter^{-1}$; tetracycline, 10 mg $liter^{-1}$; and Cm, 2 mg $liter^{-1}$ for *V. cholerae* and 20 mg $liter^{-1}$ for *Escherichia coli*.

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 with standard procedures (2). Automated DNA sequencing was carried out as described previously (43) 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.), the Vector NTI program (InforMax, North Bethesda, Md.), and the BLAST programs (1) available on the web site of the National Center for Biotechnology Information (Bethesda, Md.). Protein sequences were analyzed for the presence of motifs with the SMART program (http://smart.embl-heidelberg.de).

Cloning and sequencing of antibiotic resistance genes of *V. cholerae* O139 MO10. The previously described cosmid pSXT1 contains a ~40-kbp insert of SXT^{MO10} DNA and mediates resistance to Su, Cm, Tm, and Sm (44). A library of pSXT1 *Eco*RI fragments was constructed in pWKS30 (45). Subsequently, plasmids mediating resistance to Su, Cm, and Tm were isolated by plating the library on L-agar plates containing the respective antibiotics. One such plasmid, pATMP1, contained a 14-kbp insert that conferred resistance to Cm and Tm; another, pSUL1, contained a 1.7-kbp insert that conferred resistance to Su. Overlapping *Bam*HI, *Pvu*II, and *Pst*I fragments of pATMP1 were subcloned into pUC18, and the DNA sequences of the inserts were determined by primer walking. Additional primer walking using pSXT1 as a template was carried out to determine the sequences flanking the inserts in pATMP1 and pSUL1 on SXT^{MO10}.

Cloning and sequencing of *dfrA1* from *V. cholerae* O1 C10488. Chromosomal DNA from C10488 was partially digested with *Sau*3AI, and then fragments of ~2 to 5 kbp were isolated and ligated with *Bam*HI-digested pWKS30. The ligation mixture was electroporated into *E. coli* DH5α and plated on L-agar plates containing Tm (250 mg $liter^{-1}$) and Ap. Two plasmids mediating Tm resistance, pYL1 and pYL8, were isolated. The inserts in these two plasmids (2.77 and 3.8 kbp, respectively) were sequenced and found to overlap.

Cloning and sequencing of *aphA1* from R391. As described previously (19, 20), *Eco*RI fragments of R391 mediating Kn resistance were subcloned into pGB2 (6). One plasmid, called pRLH422, contained a single ~11-kbp *Eco*RI fragment and was used for our present studies. The DNA sequence of the ~11-kbp *Eco*RI fragment was obtained by nebulizing 20 μg of pRLH422, so as to randomly shear the DNA into fragments of 1 to 2 kbp. These fragments were blunt ended and subsequently cloned into *Sma*I-digested pUC19; 288 clones were picked and arrayed into three 96-well plates. The DNA sequence of the inserts was obtained using an Applied Biosystems ABI377 sequencer using standard sequencing protocols and primers that were designed to extend from both the 5′ and 3′ ends of the vector into the insert. The sequence data obtained were aligned into a contiguous sequence using the PhredPhrap program, and the correct alignment of the compiled sequence was confirmed by restriction mapping based on the compiled sequence.

PCR amplification. The primers used in this study are listed in Table 2 and were synthesized by the Tufts Medical School DNA Sequencing Core facility. PCRs were performed using standard reaction conditions in total volumes of 20 μl.

Nucleotide sequence accession numbers. The sequence of the antibiotic resistance gene cluster of SXT^{MO10} has been deposited in GenBank under accession no. AY034138. The sequence of the integron of SXT^{ET} has been deposited under accession no. AY035340. The sequence of the Kn resistance transposon found in R391 has been deposited under accession no. AF375956.

RESULTS AND DISCUSSION

Arrangement of antibiotic resistance genes in *V. cholerae* O139 strain MO10. We previously constructed a cosmid library with chromosomal DNA derived from O139 strain MO10, a 1992 clinical isolate from Madras, India (44). pSXT1, one of the cosmids from this library, was found to confer resistance to

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Reference or source
V. cholerae O139		
MO10	Toxigenic 1992 clinical isolate from India, SXT^{MO10+}, Su^r Tm^r Cm^r Sm^r	41
AS207	Toxigenic 1997 isolate from Calcutta, Su^r Tm^S Cm^r Sm^r	36
E712	Nontoxigenic 1994 isolate from Sri Lanka, Su^r Tm^r Cm^r Sm^r	30
2055	1998 clinical isolate from Bangladesh, Su^S Tm^S Cm^S Sm^S	21
HKO139-SXT^S	Clinical isolate from Hong Kong, Su^s Tm^S Cm^S Sm^s	47
V. cholerae O1		
CO943	El Tor 1994 clinical isolate from India, Su^r Tm^r Cm^r Sm^r	44
1811/98	El Tor 1998 clinical isolate from Bangladesh, Su^r Tm^r Cm^r Sm^r	21
C10488	El Tor 1999 clinical isolate from Bangladesh, Su^r Tm^r Cm^r Sm^r	This study
E. coli K-12		
TOP10	F′ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 endA1*	Invitrogen
DH5α	F^- *thi-1* Δ*lacU196* ϕ80*lacZ*ΔM15 *hsdR17 recA1 endA1*	
Plasmids		
pSXT1	pSuperCos1 containing a part of the SXT element encoding Cm^r Su^r Tm^r Sm^r	44
pWKS30	Ap^r pSC101 derivative	45
pATMP1	pWKS30 + 14 kbp from SXT^{MO10}, Cm^r Tm^r Sm^r	This study
pSUL1	pWKS30 + 1.7 kbp from SXT^{MO10}, Su^r	This study
pYL1	pWKS30 + 2.77 kbp from SXT^{ET}, *dfrA1*$^+$	This study
pYL8	pWKS30 + 3.8 kbp from SXT^{ET}, *dfrA1*$^+$	This study
pGB2	Spc^r pSC101 derivative	6
pRLH422	pGB2 + 11 kbp from R391, Kn^r	19, this study

Su, Cm, Tm, and Sm, indicating that the genes mediating these resistances were not randomly distributed in SXT^{MO10}. Isolation of subclones of the ~40-kbp insert from pSXT1 (as described in Materials and Methods) revealed that these resistance genes were in fact clustered together in a region of about 9.4 kbp (Fig. 1). Detailed analysis of the DNA sequence of this region along with that of flanking sequences resulted in two major findings. First, the antibiotic resistance genes appear to be part of a large transposon-like element. This element is itself a mosaic composed of other transposon-like elements and DNA sequences found in other mobile elements. Second, SXT^{MO10} contains previously identified genes (*floR, sul*II, *strA*, and *strB*) encoding resistance to Cm, Su, and Sm, respectively, and a novel gene encoding resistance to Tm.

SXT^{MO10} appears to have acquired its antibiotic resistance genes and some adjacent sequences via a transposition event(s). This event introduced a 17.2-kbp region containing all five resistance genes into *rumB*, the second gene of the *rumAB* operon. This is likely to have been a multistep process, as outlined below. Consistent with this hypothesis, the 17.2-kbp sequence is flanked both by an 8-bp direct repeat (corresponding to amino acids [aa] 76 to 78 of *rumB*) and by 16-bp imperfect inverted repeats, structures often found at the boundaries of transposons.

A role for transposition is also suggested by the presence of open reading frames (ORFs) with similarity to previous described transposases at the left end of these 17.2 kbp (Fig. 1 and Table 3). The deduced amino acid sequence of *orf1* has 39% similarity to the C terminus of a transposase found in *Pseudomonas putida* (Table 3), and the deduced amino acid sequence of *orf2* has 29% identity and 47% similarity to a transposase found in Tn*5501* and Tn*5502*, two cryptic transposons located in *P. putida* (25). The 5′ end of *orf2* is repeated downstream of *sul*II. However, despite its transposon-like features, the 17.2-kbp sequence is apparently not (or no longer) an autonomously mobile genetic element; all our attempts to mobilize the resistance genes independent of the remainder of SXT^{MO10} have failed.

The Tm resistance gene of SXT^{MO10} was mapped to subclones of pSXT1 that included a 551-bp ORF. As this ORF's deduced amino acid sequence had 37% identity and 52% similarity (Table 3) to a type VIII dihydrofolate reductase found in some *E. coli* strains (39), it was named *dfr18*, for a new gene encoding a Tm-resistant dihydrofolate reductase. *dfr18* is preceded by three ORFs, *orf3, orf4*, and *orf5*, with the same orientation as *dfr18*. The deduced amino acid sequences of *orf3* and *orf4* do not have similarities to any known proteins, but the deduced amino acid sequence of *orf5* has 44% identity and

TABLE 2. DNA sequences of the oligonucleotides used in this study

Primer	Locus (direction)[a]	Nucleotide sequence (5′ to 3′)
INT1	*int* (+)	GCTGGATAGGTTAAGGGCGG
INT2	*int* (−)	CTCTATGGGCACTGTCCACATTG
FLOR-F	*floR* (+)	TTATCTCCCTGTCGTTCCAGCG
FLOR-B	*floR* (−)	TCGTCGAACTCTGCCAAATG
SUL2-F	*sulII* (+)	AGGGGGCAGATGTGATCGAC
SUL2-B	*sulII* (−)	TGTGCGGATGAAGTCAGCTCC
STRA-F	*strA* (+)	TTGATGTGGTGTCCCGCAATGC
STRA-B	*strA* (−)	CCAATCGCAGATAGAAGGCAA
TMP-F	*dfr18* (+)	TGGGTAAGACACTCGTCATGGG
TMP-B	*dfr18* (−)	ACTGCCGTTTTCGATAATGTGG
TMP3	*orfA* (+)	CATGCTGTTTCTCGACGGTG
TMP4	*orf6* (−)	GATCCGATCTGTTTGTTCAG
LEND4	*orf1* (+)	CCTTTGGTTACACATTCGC
LEFTF3	*rum′B* (−)	GGTGCCATCTCCTCCAAAGTGC
RUMA	Intergenic	CGAGCAATCCCCACATCAAG
YL6	*orf73* (+)	TGTGGAACGGCTTTCTGACG
YL3	*orfC5A* (+)	CGTTGGTTTGGGGTAACACC

[a] +, oligonucleotides corresponding to the coding strand (forward primer), −, oligonucleotides corresponding to the noncoding strand (backward primer).

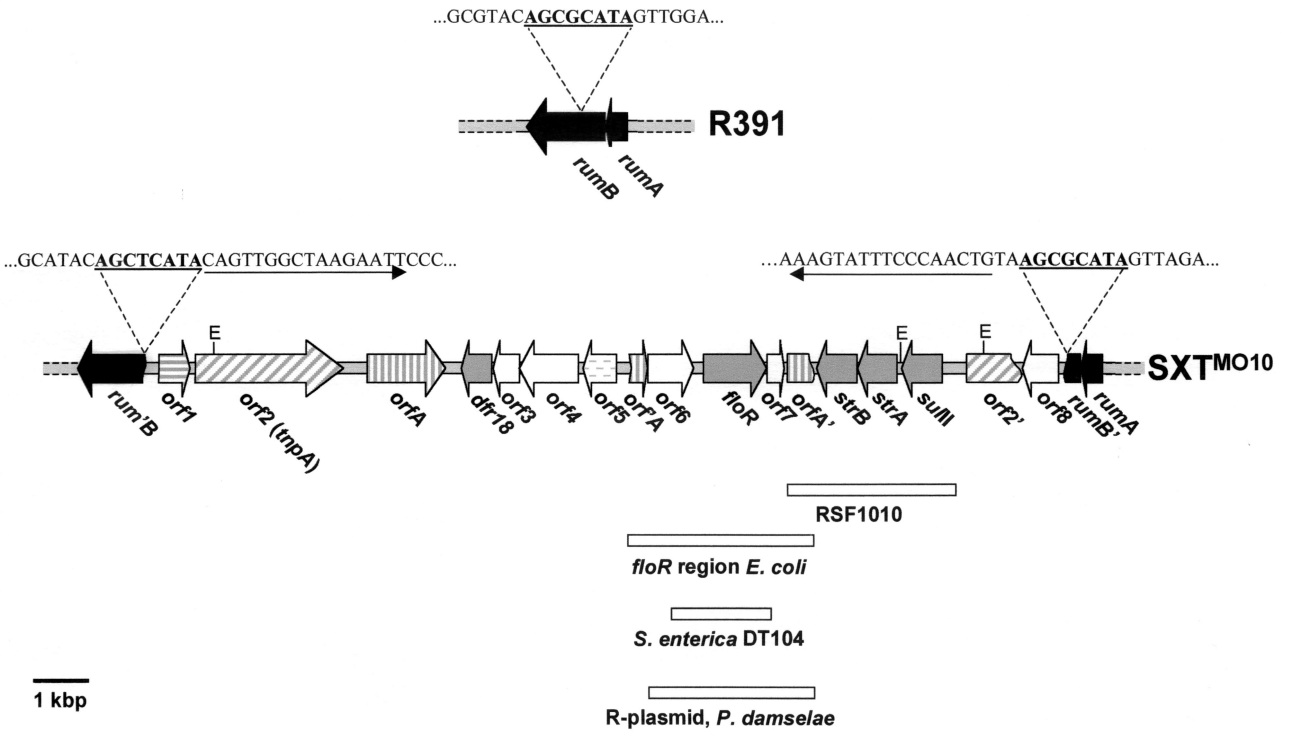

FIG. 1. Organization of the antibiotic resistance gene cluster in SXT^{MO10}. The SXT^{MO10} genes mediating resistance to antibiotics, *dfr18, floR, strA, strB*, and *sulII*, are represented by gray arrows, and genes with similarity to transposases (*orf1, orf2*, and *orfA*) are represented by hatched arrows. Genes encoding hypothetical proteins similar to known proteins are shown as horizontal hatches, and genes encoding hypothetical proteins dissimilar to known proteins are shown in white. Genes *rumA* and *rumB* are in black. The *rumAB* operon of R391 is presented above the SXT^{MO10} antibiotic resistance gene region. The sequence in *rumB* which is repeated in SXT^{MO10} is in bold and underlined; the flanking imperfect repeat (IR) sequences in SXT^{MO10} are marked by arrows. Also indicated are the *Eco*RI sites (E) used for construction of pATMP1 and pSUL1. Regions of nucleotide sequence identity to other published nucleotide sequences are represented by boxes.

60% similarity (Table 3) to a chromosomal *Pseudomonas aeruginosa* deoxycytidine triphosphate deaminase (37). Whether *orf5* encodes a functional deaminase remains to be studied. These four genes are bracketed by the previously described *orfA* (7). A complete copy of *orfA* lies downstream of *dfr18*, while a 5′-truncated copy of *orfA* lies upstream of *orf5* (Fig. 1 and Table 3). An identical full-length *orfA* was found by Cloeckaert et al. in a plasmid from an *E. coli* isolate (7). The predicted OrfA amino acid sequence has been noted to have some similarity to a putative transposase from *Pseudomonas pseudoalcaligenes* (12). It seems likely that *orfA* plays some role in promoting the acquisition and loss of antibiotic resistance genes, since *orfA* or fragments of *orfA* are closely linked to antibiotic resistance genes in several instances (7, 22, 35). The molecular mechanism(s) by which *orfA* acts to promote gain or loss of genes remains to be explored.

In two prior cases, *orfA* or *orfA* fragments have been found associated with *floR* (7, 22). This is the case in SXT^{MO10} as well (Fig. 1). In SXT^{MO10}, *floR* is found close to the 3′ end of the 5′-truncated *orfA*, preceded by a putative ORF (*orf6*) of unknown function. FloR is thought to be an export protein which mediates resistance to Cm and florfenicol. This gene has been found in plasmids derived from *E. coli* isolates from cattle (7), in the chromosome of the multidrug-resistant *Salmonella enterica* serovar Typhimurium phagetype DT104 (4) and in an R-plasmid derived from the fish pathogen *Photobacterium damselae* subsp. *piscida* (22). As expected, in-frame deletion of *floR* from SXT^{MO10} resulted in cells that were no longer resistant to Cm (J. Beaber and B. Hochhut, unpublished observations), confirming that *floR* is required for resistance to Cm. In SXT^{MO10}, *floR* is followed by a short putative ORF (*orf7*) that includes a region with similarity to the helix-turn-helix (HTH) motif of LysR family transcriptional regulators, and another incomplete copy of *orfA* that is deleted in its 3′ end. The two incomplete copies of *orfA* that bracket *floR* together do not constitute a full-length *orfA*. Comparative DNA sequence analysis revealed extensive nucleotide identity to the *floR* loci in *E. coli* isolates and *P. damselae* (Fig. 1). The genes *strA, strB*, and *sul*II, which follow *orfA′*, are identical to previously described resistance genes and are found on several plasmids, including RSF1010 (35). They encode a sulfonamide-resistant dihydropterate synthase (*sul*II) and an aminoglycoside phosphotransferase (*strAB*).

Distribution of SXT^{MO10} antibiotic resistance genes in related SXT elements from *V. cholerae* O1 and O139 strains. Since the discovery of SXT^{MO10} in isolates from the initial O139 outbreak in 1992, closely related constins have been detected in many *V. cholerae* isolates of both the O1 and O139 serogroups. These related constins, like SXT^{MO10}, are integrated into *prfC* (21); however, these elements do not confer the same antibiotic resistances as SXT^{MO10}. For example, many recent O139 clinical isolates from Asia were found to be

TABLE 3. Gene products with sequence similarity to the antibiotic resistance gene cluster in SXT^{MO10}

Coding region[a]	Gene name	Length (aa)	Closest similarity	Accession no.	% Identity/range[b]
1–1044 (−)	*rum′B*		RumB R391 (C terminus)	862633	
1457–2047	*orf1*	197	Transposase, *P. putida*	4754812	39/188 of 530
2164–5061	*orf2* (*tnpA*)	966	Transposase, *P. putida*	7465523	29/956
5559–7049	*orfA*	497	Putative transposase, *E. coli*	10312101	99/496
7416–7967 (−)	*dfr18*	184	Dihydrofolate reductase type VIII	2833495	37/157
7984–8526 (−)	*orf3*	181	No homology	NA[c]	NA
8529–9692 (−)	*orf4*	388	No homology	NA	NA
9710–10435 (−)	*orf5*	294	Deoxycytidine triphosphate deaminase, *P. aeruginosa*	9949625	44/208
10695–11024	*orf′A*	110	Putative transposase, *E. coli* (3′ end, aa 388–497)	10312101	
11058–11939	*orf6*	294	No homology	NA	NA
12159–13370	*floR*	404	FloR (florfenicol exporter), *E. coli*	10312100	99/404
13401–13703	*orf7*	101	Putative transcriptional regulator (LysR family), *P. aeruginosa*	11352177	51/74
13818–14354	*orfA′*	179	Putative transposase, *E. coli* (5′ end, aa 1–135)	10312101	
14332–15165 (−)	*strB*	278	Aminoglycoside phosphotransferase	420965	100/278
15168–15968 (−)	*strA*	267	Aminoglycoside phosphotransferase	420964	100/267
16032–16844 (−)	*sul*II	271	Dihydropteroate synthase transposase, *P. putida* (5′ end)	1075456	100/271
17315–18674	*orf2′* (*tnpA′*)			7465523	24
18399–19118 (−)	*orfB*	240	MutI, *V. cholerae*	127554	51/173 (217–345 of 563)
19308–19540 (−)	*rumB′*		RumB R391 (N terminus)	862633	
19551–19997 (−)	*rumA*	149	RumA R391	862632	98/149

[a] Genes encoded on the minus strand are indicated with (−).
[b] The number of amino acids in a contiguous stretch from which the identity was calculated is shown. The length of the similar protein is also presented.
[c] NA, not applicable.

sensitive to Su and Tm (28). We analyzed the genetic basis for this sensitivity in two O139 clinical isolates, strain 2055 from Bangladesh and strain HKO139-SXT^S from Hong Kong. PCR assays designed for amplification of internal sequences of *dfr18, floR, strA*, and *sulII* from these strains failed, whereas a PCR amplification of int_{SXT}, a signature sequence of an SXT-related constin, was successful (Table 4). PCR assays utilizing primers that flank the antibiotic resistance genes in SXT^{MO10} facilitated the mapping of the borders of the DNA missing in strains 2055 and HKO139-SXT^S. Using chromosomal DNA from either strain as the template, with primer pair LEND4 and RUMA, we obtained a product of ~3.3 kbp, and with primer pair LEFTF3 and RUMA, we amplified a 4-kbp product (Fig. 2). In contrast, in MO10 these primer pairs flank sequences of 18.5 and 19.2 kbp.

The DNA sequence of the 3.3-kbp fragment was partially determined. As in MO10, the reading frame of *rumB* is interrupted in these strains, but by a much smaller insert encompassing *orf1, orf2′*, and *orf8*. This genetic structure suggests that deletion mediated by homologous recombination between the two identical 5′ ends of *orf2* that bracket the resistance gene cluster in SXT^{MO10} may have rendered these strains sensitive to antibiotics (Fig. 2). An alternative possibility is that these antibiotic-sensitive O139 strains never carried any of the resistance genes and that their constins represent a precursor of SXT^{MO10}. Since the *rum* operon is interrupted in both types of elements, the latter possibility seems less likely. In either case, the lack of the ~15.2-kbp fragment from these antibiotic-sensitive O139 strains has not rendered their SXT-like elements deficient for transfer (data not shown).

Other recent int_{SXT}-containing O139 isolates, such as the 1996 Calcutta isolate AS207, have been found to be resistant to Cm, Su, and Sm but sensitive to Tm (21, 27). Using AS207 DNA as the template, we were able to amplify *floR, strA*, and *sulII* by PCR (Table 4). Southern hybridization indicated that the arrangement of these genes was similar in AS207 and in MO10 (data not shown). However, both a PCR assay (Table 4) and a Southern hybridization assay (not shown) indicated that AS207 lacked *dfr18*. The precise borders of the deletion including *dfr18* in the AS207 constin are discussed below.

After the initial spread of *V. cholerae* O139 on the Indian subcontinent in 1993, clinical isolates of *V. cholerae* O1 El Tor from this region were found to be resistant to the same antibiotics, Su, Sm, Tm, and Cm, as O139 strains. We analyzed the genes encoding these resistances in three El Tor strains, CO943, 1811, and C10488, isolated in different years and from different locations on the Indian subcontinent (Table 1). As in O139 strain MO10, the resistance determinants in these strains were part of a constin designated SXT^{ET}, that is very similar but not identical to SXT^{MO10} (21, 44; data not shown). Using chromosomal DNA from these strains as templates for PCR,

TABLE 4. Distribution of antibiotic resistance genes in *V. cholerae* isolates containing int_{SXT}[a]

Strain	Serogroup	Detection[b]				
		dfr18	*dfrA1*	*floR*	*strA*	*sul*II
MO10	O139	+	−	+	+	+
HKO139-SXT^S	O139	−	−	−	−	−
2055	O139	−	−	−	−	−
AS207	O139	−	−	+	+	+
E712	O139	−	+	+	+	+
CO943	O1	−	+	+	+	+
1811	O1	−	+	+	+	+
C10488	O1	−	+	+	+	+

[a] All strains tested were positive for int_{SXT} in a PCR assay.
[b] Symbols: +, listed gene detected by a PCR assay and by a Southern hybridization experiment; −, no detection of the gene.

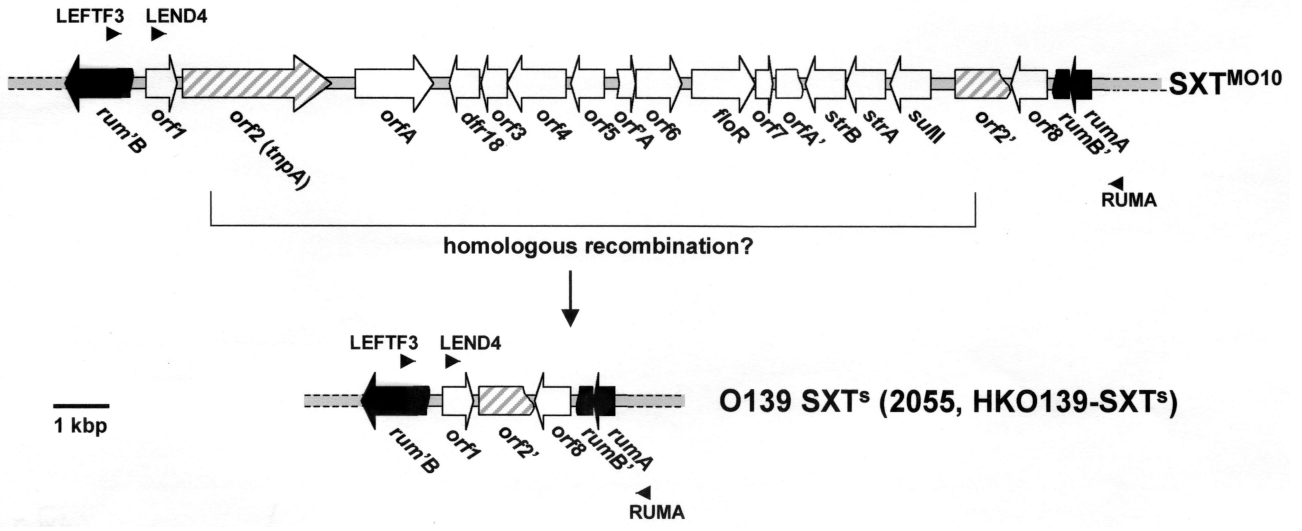

FIG. 2. Organization of the region containing antibiotic resistance genes in SXT^{MO10} and in *V. cholerae* O139 strains sensitive to Tm, Su, and Cm. The gene order found in strains 2055 and HKO139-SXT^{S} (bottom) is compared to that of SXT^{MO10} (top). Homologous recombination between the identical sequences in *orf2* and *orf2′* may have resulted in loss of the antibiotic resistance genes. Also shown are the primers (LEFTF3, LEND4, and RUMA) used to amplify this region in 2055 and HKO139-SXT^{S}.

products corresponding to internal regions of *floR, strA*, and *sulII* were amplified (Table 4). Southern hybridization experiments indicated that the organization of these genes in SXT^{ET} is identical to that in SXT^{MO10} (data not shown). To our surprise, despite their resistance to Tm, these El Tor isolates were found by PCR (Table 4) and Southern hybridization (not shown) not to harbor *dfr18*.

PCR primers (TMP3 and TMP4, Fig. 3) which anneal to sequences that flank *dfr18* in SXT^{MO10} were used to define the extent of the region missing from SXT^{ET}. Using these primers and MO10 chromosomal DNA as the template, a PCR product with the expected size of 5.35 kbp was obtained, whereas with C10488 chromosomal DNA as the template, a product of 1.3 kbp was obtained. The DNA sequence of this 1.3-kbp PCR product revealed that in addition to *dfr18, orf3, orf4*, and *orf5* were also absent in C10488 (Fig. 3). Furthermore, in C10488, a complete copy of *orfA* is followed by *orf6* and *floR*, whereas

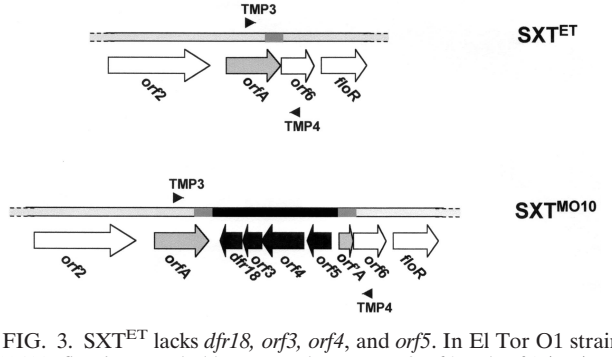

FIG. 3. SXT^{ET} lacks *dfr18, orf3, orf4*, and *orf5*. In El Tor O1 strain C10488, *floR* is preceded by a complete copy of *orf6* and *orfA* (top). In contrast, in SXT^{MO10}, there is a duplication of 640 bp (dark gray boxes) that flanks the genes *dfr18, orf3, orf4*, and *orf5* (black). The locations of primers (TMP3 and TMP4) used for amplification of this area are also shown.

in MO10, a complete copy of *orfA* is located next to *dfr18* and only a 5′-end-truncated copy of *orfA* is found next to *orf6* (Fig. 3). The 3.34-kbp "insert" that includes the genes *dfr18, orf3, orf4*, and *orf5* and that distinguishes SXT^{MO10} from the constin present in C10488 is flanked by a 640-bp duplication (Fig. 3). This repeated DNA sequence encompasses the 3′ end of *orfA* and the first 205 bp of *orf6*. A PCR showed that the same sequences were also missing from the constins in the other two El Tor Tm^{r} strains, CO943 and 1811/98, as well as in the constin in the Tm^{s} O139 strain AS207 discussed above. We have no direct evidence of the mechanism by which these additional genes were acquired by SXT^{MO10} or, alternatively, lost from the C10488 constin. However, given the presence of the duplicated 640-bp sequence, homologous recombination probably played some role in the loss or acquisition of these four genes.

***dfrA1* mediates Tm resistance in *V. cholerae* O1 constin.** Although the constin in strain C10488 lacked *dfr18*, we strongly suspected that the determinant of Tm resistance in this strain would be part of SXT^{ET}, since the Su, Sm, Cm, and Tm resistance determinants were cotransferred by C10488 (data not shown). We constructed a plasmid library with insert DNA derived from C10488 chromosomal DNA to isolate the Tm resistance determinant(s) from this strain. We identified two recombinant plasmids (pYL1 and pYL8) that allowed their host cells to grow on media containing Tm. Determination of their respective insert DNA sequences revealed that they contained overlapping inserts and that the overlap included an ORF with nucleotide sequence identity to the previously described gene *dfrA1* (Fig. 4) (14). *dfrA1* encodes a trimethoprim resistance dihydrofolate reductase which until now has been found exclusively as a cassette within class 1 and 2 integrons (11, 32). Instead, *dfrA1* from C10488 appears to be part of a novel (class 4) type of integron; 271 bp upstream of the *dfrA1* cassette was a gene of 320 codons whose deduced amino acid

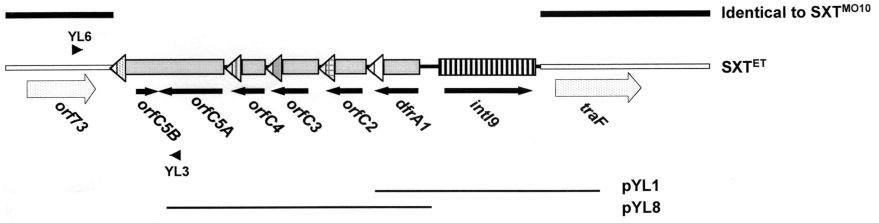

FIG. 4. Organization of the integron in SXT^{ET} constin. The five cassettes found in the SXT^{ET} integron are shown. The *attC* sites are represented by triangles, and ORFs are represented below the cassettes as arrows. Also shown are the genes *traF* and *orf73*. DNA sequences identical to SXT^{MO10} are shown as black lines. The insert DNA in pYL1 and pYL8 is shown below. The positions of primers YL6 and YL3, used to amplify the upstream boundary of the integron insertion in SXT^{ET}, are also indicated.

sequence showed similarity to the site-specific recombinases found in integrons and which has been named *intI9*. Its predicted product, IntI9, shows 53% identity to IntI2* (a 325-amino-acid protein obtained through readthrough of the stop codon at position 178 in *intI2* [accession no. NP_065308]), a putative integrase of the class 2 resistance integrons. The paradigm of class 2 integrons is found on Tn*7*. The second closest relative of IntI9 is SpuIntIA, the *Shewanella putrefaciens* chromosomal integron integrase (47% identity) (34). *dfrA1* and *intI9* are oriented in opposite directions, an arrangement characteristic of integrons. Furthermore, the DNA sequence of the *dfrA1* cassette is 99.8% identical to the *dfrA1* cassette of class 1 and 2 resistance integrons.

The sequence downstream of the *dfrA1* cassette did not show similarity to any known genes. However, analysis of this sequence revealed the presence of four putative consecutive integron cassettes (Fig. 4). Cassettes 2, 3, and 4 each carry a single ORF, while cassette 5 contains two ORFs in opposite orientation. The putative product of *orfC2* (142 aa) is predicted to be located in the cytoplasmatic membrane. The deduced amino acid sequence of *orfC3* (136 aa) contains a region with similarity to an Xre-type HTH motif and is predicted to the membrane associated. Finally, the product of *orfC5A* (233 aa) has an AraC-type HTH motif, while the putative product of *orfC5B* (82 aa) contains a domain conserved among bleomycin resistance proteins. Although integrons were originally described as systems to capture antibiotic resistance genes, analysis of superintegron cassettes has revealed that many of the genes contained therein are of unknown function (32, 34).

As seen for the cassettes carried in the multiresistance integrons, the *attC* sites carried by the SXT^{ET} integron cassettes are extremely different in length (58 to 99 bp) and sequence. Interestingly, the *attC* site of cassette 2 is almost identical to the *attC* site of the first cassette of the *S. putrefaciens* CIP 69.34 superintegron (accession no. AF324211) (34), while the genes carried in both cassettes are unrelated. In contrast, the *attC* sites of the other three cassettes do not show any significant homology (<50% identity) with the *attC* sites found either in previously described resistance cassettes or in any of the superintegron cassettes, including those of the *V. cholerae* superintegron.

In our ongoing research, we are determining the complete nucleotide sequence of SXT^{MO10}. We took advantage of the partially completed sequence to determine where the *dfrA1*-containing integron is located in the C10488 constin. Downstream of *intI9* in the insert of pYL1 was a region with near nucleotide sequence identity to SXT^{MO10} (Fig. 4). In SXT^{MO10}, this region encodes a putative gene (*traF*) thought to be required for pilus assembly; it is located about 70 kbp away from the resistance gene cluster. Unlike the insert in pYL1, the sequence of the pYL8 insert did not show any similarity to the sequence of SXT^{MO10} (Fig. 4).

To identify the upstream boundary of the apparent insertion of the *dfrA1*-containing integron, we designed PCR primers to amplify the junction between this integron-like element and predicted upstream sequences in SXT^{MO10} (Fig. 4). With the primer pair YL6/YL3, we amplified a product of ~1 kbp with C10488 chromosomal DNA as a template. Sequence analysis of this PCR product, combined with the sequences of the pYL1 and pYL8 inserts, revealed that relative to SXT^{MO10}, an insert of 4.77 kbp is present in SXT^{ET} between *traF* and an ORF of unknown function, *orf73* (Fig. 4). Examination of the borders of the integron in SXT^{ET} did not reveal sequences such as inverted or direct repeats that might suggest the mechanism by which this integron was acquired. However, we noticed that the divergence between the sequences of SXT^{MO10} and SXT^{ET} downstream of *orf73* coincided exactly with the core site sequence in *attC* of cassette 5. In this region the MO10 sequence does not show any of the *attC* site structural characteristics apart from a conserved CGTT sequence, which is precisely located at the beginning of the identity with the SXT^{ET} sequence. Integrase-mediated recombination between *attC* sites and noncanonical sites, known as secondary sites of consensus GWTMW (15), has been reported several times (15, 16, 33). This suggests that this boundary of the integron likely corresponds to a recombination between the *attC* site of cassette 5 and the sequence AACGTTCTGC (bases corresponding to bases fitting the secondary site consensus shown above are underlined) of the SXT backbone. To our knowledge, this is the first evidence of such an event to explain the 3′ end of a cassette array in an integron. The only natural case of likely recombination between an *attC* site and a secondary site described so far was the integration of a single *aadB* cassette, not an integron, into an RSF1010 plasmid (33).

Like C10488, the other two El Tor strains we studied, CO943 and 1811, also contained a 4.77-kbp sequence inserted between *orf73* and *traF*. Insertion of a *dfrA1*-containing integron into this locus was not limited to the constins found in El Tor strains. We identified a nontoxigenic O139 isolate, E712, that also contained this insertion (Table 4). In fact, like SXT^{ET}, the E712 constin also lacked the 3.34-kbp region containing

dfr18, present in SXT^{MO10} (Table 4), suggesting that the E712 constin was very similar (or identical) to the El Tor constin.

***aphAI* in R391 is part of a transposon.** Although SXT^{MO10} and R391 are closely related constins, they encode different sets of antibiotic resistances. Cells carrying R391 are sensitive to Cm, Tm, Su, and Sm but resistant to Kn. As expected, PCR assays and Southern analyses revealed that R391 does not carry any of the resistance genes or putative transposase genes encoded in SXT^{MO10} (20; data not shown). Also, R391 contains an intact *rumAB* operon (19, 24). This operon encodes proteins that are phylogenetically related to a superfamily of novel error-prone DNA polymerases found in all three kingdoms of life (46). While R391 complements the DNA repair functions encoded by the *umuDC* operon in *E. coli* strains missing these genes (19), SXT^{MO10} failed to complement a Δ*umuDC* strain (data not shown), confirming the inactivation of *rumB*.

DNA sequence analysis of the ~11-kbp *Eco*RI fragment carrying the R391 Kn resistance determinant revealed on ORF some ~4 kbp from the *rumAB*$_{R391}$ locus that is identical to the previously described *aphAI* gene, which encodes an aminoglycoside phosphotransferase (29). Immediately 5′ and 3′ of *aphAI*, we identified two copies of IS*26* in opposite orientations (data not shown), indicating that *aphAI* is part of a novel transposon. Interestingly, linkage of *aphAI* with IS*26* is also found in the multiresistance plasmid pSP9351 from *P. damselae* (23); however, the organization of IS*26* relative to *aphAI* differs between pSP9351 and R391. Taken together, our data indicate that antibiotic resistance genes can be added to SXT-like constins at several locations and via different mechanisms.

Conclusions. SXT-related constins constitute an important group of transmissible genetic elements that have contributed to the spread of resistance to antimicrobial agents in clinical isolates of *V. cholerae* from Asia. Our surveys of *V. cholerae* O139 and O1 clinical isolates from this region indicate that the great majority of post-1993 isolates contain an SXT-related element integrated on the large *V. cholerae* chromosome at *prfC*. Thus far, all of the elements tested are self-transmissible and encode Int$_{SXT}$, the defining features of SXT-related constins. Although the genetic determinants of the transfer and integration functions of these related elements appear to be nearly identical, in the current study we found that the antibiotic resistance genes in these elements differed. In the SXT constin found in the original 1992 O139 outbreak strains (SXT^{MO10}), as exemplified by MO10 (and found in other isolates as well), the antibiotic resistance genes were all clustered together within a ~17-kbp composite transposon-like structure. In contrast, in the SXT^{ET} constin found in the reemerged (post-1993) El Tor O1 strains, this cluster is missing a 3.3-kbp segment that includes the novel *dfr18* found in SXT^{MO10}. Instead, SXT^{ET} contains a novel integron-like structure that includes *dfrA1*, located ~70 kbp away from the other antibiotic resistance genes in this constin. Finally, R391 contains a transposon-associated Kn resistance gene located ~3.5 kbp away from the site where the composite transposon-like element apparently inserted in SXT^{MO10}. The differences in the antibiotic resistance genes in SXT-related constins suggest that these genes are not intrinsic features of this family of constins; they appear to have inserted themselves on these elements as a way to become transmissible through bacterial populations.

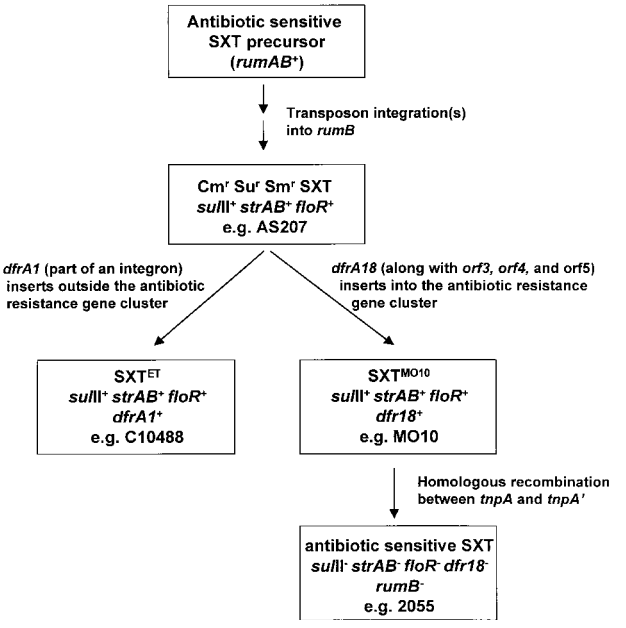

FIG. 5. Model for antibiotic resistance gene flux in the SXT family of constins.

Selective pressure to become and remain resistant to antibiotics does not seem to be the only explanation for the dissemination and persistence of SXT-related constins in Asian *V. cholerae*. This is clear from the absence of antibiotic resistance genes from the SXT-like constins found in many recent O139 isolates, such as strain 2055 analyzed in this study. The advantage(s) conferred by constins lacking resistance genes remains to be elucidated.

A plausible scheme outlining the steps in the acquisition and loss of antibiotic resistance genes in the *V. cholerae* derived SXT family of constins is shown in Fig. 5. First, in one or several steps, a transposon(s) that included *sulII, strAB*, and *floR* inserted into *rumB*, a gene that is intact in R391, an SXT-related constin. Then, the resulting Su^r, Sm^r, and Cm^r constin (such as was found in O139 strain AS207) could have become Tm^r by acquiring either the novel integron containing *dfrA1*, to give rise to SXT^{ET}, or *dfr18, orf3, orf4*, and *orf5*, to give rise to SXT^{MO10}. This latter event likely depended on *orfA* (by an unknown mechanism), since *orfA* is associated with antibiotic resistance genes in several instances. Subsequently, the Su^r, Sm^r, and Cm^r constin could have undergone a deletion event, likely mediated by homologous recombination, to give rise to constins that lack antibiotic resistance genes such as those found in O139 strains 2055 and HKO139-SXT^S. Even though SXT^{MO10} was the first SXT-family constin that we identified (from a 1992 O139 isolate) and we did not detect SXT^{ET} in O1 strains until 1994, given the differences in the antibiotic resistance genes between these two constins, it seems unlikely that SXT^{MO10} is an immediate precursor of SXT^{ET}. Rather, SXT^{ET}, the constin found in most recent O1 isolates, seems to have arisen independently of SXT^{MO10}. We detected an SXT^{ET}-like element in an O139 isolate (E712), indicating that SXT^{ET} is not limited to the *V. cholerae* O1 serogroup. Additionally, we found SXT^{ET} (or at least very similar ele-

ments) in *Providencia alcalifaciens* isolates from patients in Bangladesh (data not shown). This suggests a recent gene transfer between *V. cholerae* and *P. alcalifaciens*. Finally, although SXT family constins are present in virtually all clinical *V. cholerae* isolates from Asia, these elements are a relatively recent addition to the *V. cholerae* genome. They are not present in seventh-pandemic *V. cholerae* isolates, as exemplified by their absence from the genome of N16961, the type strain used for determination of the complete nucleotide sequence of the *V. cholerae* chromosomes by the Institute for Genome Research. The bacterial species that donated SXT family constins to Asian *V. cholerae* remains to be determined.

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