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* El 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 SXTMO10, 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 (suIII), 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, SXTET, does not contain the same resistance genes as SXTMO10. 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 transcription 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 sulfamethoxazole (Su), trimethoprim (Tm), chloramphenicol (Cm), and low levels of streptomycin (Sm). In MO10, a 1992 clinical O139 isolate, the genes encoding these...
resistances were found to be located on a novel transmissible genetic element designated the SXT element (referred to here as SXT\textsuperscript{MO10}) (44).

Though it is self-transmissible, an autonomously replicating extrachromosomal form of SXT\textsuperscript{MO10} has not been isolated; instead, this $\sim$100-kbp element is always integrated into the 5′ end of the chromosomal gene prfC. SXT\textsuperscript{MO10} encodes an integrase related to the $\lambda$ 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 nonrepli-
cating form of the element is believed to be a requisite inter-

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After the extensive cholera outbreaks caused by \textit{V. cholerae} O139 strains, El Tor O1 \textit{V. cholerae} strains reemerged in 1994 as the predominant cause of cholera on the Indian subconti-
nent. 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, Sm, and Tc (48). The corresponding resistance genes were found to be located in a constin (designated here SXT\textsuperscript{ET}) that is closely related but not identical to SXT\textsuperscript{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 anal-

yses have revealed the presence of an SXT\textsuperscript{MO10}-like element integrated into \textit{prfC} in these strains, indicating that they still harbor constins related to SXT\textsuperscript{MO10} (21).

SXT-like elements are not unique to \textit{V. cholerae} O139. For example, the Inc element R391 that mediates kanamycin (Kn) and mercuric resistance, originally derived from a South African \textit{Providencia retgerii} isolate (8), is functionally and geneti-
cally related to SXT\textsuperscript{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\textsuperscript{MO10} and compared them to those of other SXT constins. The SXT\textsuperscript{MO10} resistance genes are embedded in a $\sim$17.2-kbp composite transposon-like element that interrupts the SXT-encoded \textit{numAB} operon. A deletion event, likely mediated by recombination between du-
luplicated sequences in this region, accounts for the Su and Tm sensitivity of recent O139 isolates. In SXT\textsuperscript{ET}, unlike in SXT\textsuperscript{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\textsuperscript{ET}. By

comparison, the Kn resistance gene in R391 is found to be part of a transposon containing IS26 elements that is located $\sim$3 kbp 5′ to the R391 \textit{numAB} 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 $\sim$70°C in LB broth containing 20% (vol/vol) glycerol. Antibiotics were used at the following concentrations: ampicillin (Ap), 100 mg liter$^{-1}$; Km, 50 mg liter$^{-1}$; Sa, 160 mg liter$^{-1}$; Tc, 32 or 250 mg liter$^{-1}$; tetracycline, 10 mg liter$^{-1}$; and Km, 2 mg liter$^{-1}$ for \textit{V. cholerae} and 20 mg liter$^{-1}$ for \textit{Escherichia coli}.

Molecular biology procedures. Plasmid DNA was prepared using the Qiaprep Spin Miniprep Kit (Qiagen, Valencia, Calif.), and chromosomal DNA was iso-

alized 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 pre-

viously (43) at the Tufts Medical School DNA Sequencing Core Facility. Compu-

ter analysis of DNA sequences was performed with the MacVector and As-

ssemblyLIGN 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 \textit{V. cholerae} O139

MO10. The previously described cosmids pSXT1 contains a $\sim$40-kbp insert of SXT\textsuperscript{MO10} DNA and mediates resistance to Su, Cm, Tm, and Sm (44). A library of pSXT1 EcoRI 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, pATM1, 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 BamHI, PvuII, and PstI fragments of pATM1 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 pATM1 and pSUL1 on SXT\textsuperscript{MO10}.

Cloning and sequencing of dfrA1 from \textit{V. cholerae} O1 C10488. Chromosomal DNA from C10488 was partially digested with Sau3A1, and then fragments of $\sim$2 to 5 kbp were isolated and ligated with BamHI-digested pWKS30. The ligation mixture was electroporated into \textit{E. coli} DH5$\alpha$ and plated on L-agar plates containing Tm (250 mg liter$^{-1}$) and Ap. Two plasmids mediating Tm resistance, pYLI 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 aphA II from R391. As described previously (19, 20), EcoRI fragments of R391 mediating Kn resistance were subcloned into pGB2 (6). One plasmid, called pRHI422, contained a single $\sim$11-kbp EcoRI fragment and was used for our present studies. The DNA sequence of the $\sim$11-kbp EcoRI fragment was obtained by nuclease 20 mg pLH422, so as to randomly shear the DNA into fragments of 1 to 2 kbp. These fragments were blunted ended and subsequently cloned into Smal-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 pro-

tocols 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 Phred/Phrap 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

$\mu$L.

Nucleotide sequence accession numbers. The sequence of the antibiotic resis-
tance gene cluster of SXT\textsuperscript{MO10} has been deposited in GenBank under accession

no. AY034138. The sequence of the integron of SXT\textsuperscript{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 \textit{V. cholerae}

O139 strain MO10. We previously constructed a cosmids 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

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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 \textit{rnumB}, the second gene of the \textit{rumAB} operon. This likely is 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 \textit{rnumB}) 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 kb (Fig. 1 and Table 3). The deduced amino acid sequence of \textit{orf1} has 39\% similarity to the C terminus of a transposase found in \textit{Pseudomonas putida} \textit{pstuDa} (Table 3), and the deduced amino acid sequence of \textit{orf2} has 29\% identity and 47\% similarity to a transposase found in \textit{Tn5501} and \textit{Tn5502}, two cryptic transposons located in \textit{P. putida} (25). The 5' end of \textit{orf2} is repeated downstream of \textit{suII}. 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 \textit{SXT MO10} have failed.

The Tm resistance gene of \textit{SXT MO10} was mapped to subclones of \textit{pSXT1} that included a 551-bp ORF. As this ORF is homologous to \textit{mrr} from \textit{SXT ET}, \textit{strA} was subcloned into \textit{pSXT1} that included a 551-bp ORF. As this ORF is homologous to \textit{mrr} from \textit{SXT ET}, \textit{strA} was subcloned into \textit{pSXT1} that included a 551-bp ORF.

\begin{table}
\centering
\caption{Bacterial strains and plasmids used in this study}
\begin{tabular}{lll}
\hline
Strain or plasmid & Relevant genotype or phenotype & Reference or source \\
\hline
\textit{V. cholerae} O139 & & \\
MO10 & Toxigenic 1992 clinical isolate from India, \textit{SXT^{MO10}-}, \textit{Su' Tm'} \textit{Cm'} \textit{Sm'} & 41 \\
AS207 & Toxigenic 1997 isolate from Calcutta, \textit{Su'} \textit{Tm'} \textit{Cm'} \textit{Sm'} & 36 \\
E712 & Nontoxigenic 1994 isolate from Sri Lanka, \textit{Su'} \textit{Tm'} \textit{Cm'} \textit{Sm'} & 30 \\
2055 & 1998 clinical isolate from Bangladesh, \textit{Su'} \textit{Tm'} \textit{Cm'} \textit{Sm'} & 21 \\
HK0139-SXT & Clinical isolate from Hong Kong, \textit{Su'} \textit{Tm'} \textit{Cm'} \textit{Sm'} & 47 \\
\textit{V. cholerae} O1 & & \\
CO943 & El Tor 1994 clinical isolate from India, \textit{Su'} \textit{Tm'} \textit{Cm'} \textit{Sm'} & 44 \\
1811/98 & El Tor 1998 clinical isolate from Bangladesh, \textit{Su'} \textit{Tm'} \textit{Cm'} \textit{Sm'} & 21 \\
C10488 & El Tor 1999 clinical isolate from Bangladesh, \textit{Su'} \textit{Tm'} \textit{Cm'} \textit{Sm'} & This study \\
\textit{E. coli} K-12 & & \\
TOP10 & F' \textit{mcrA} \textit{(mar-hsdRMS-mcrBC)} \textit{ΔlacZΔM15 ΔacX74 recA1 endA1} & Invitrogen \\
DH5α & F' \textit{thi-1 ΔlacU166 Δ88lacZΔM15 hisdR17 recA1 endA1} & \\
\textbf{Plasmids} & & \\
pSXT1 & \text{pSuperCos1 containing a part of the SXT element encoding Cm' Su' Tm'} \textit{Cm'} \textit{Sm'} & 44 \\
pWK30 & \text{Ap' pSC101 derivative} & 45 \\
pATMP1 & \text{pWK30 + 14 kbp from \textit{SXT^{MO10}}, Cm' Tm'} \textit{Cm'} \textit{Sm'} & This study \\
pSUL1 & \text{pWK30 + 1.7 kbp from \textit{SXT^{MO10}}, Su'} & This study \\
pYL1 & \text{pWK30 + 2.77 kbp from \textit{SXT^{ET}}, dfrA1} & This study \\
pYL8 & \text{pWK30 + 3.8 kbp from \textit{SXT^{ET}}, dfrA1} & This study \\
pGB2 & \text{Spe' pSC101 derivative} & 6 \\
pRLH422 & \text{pGB2 + 11 kbp from R391, Kn'} & 19, this study \\
\hline
\end{tabular}
\end{table}

\begin{table}
\centering
\caption{DNA sequences of the oligonucleotides used in this study}
\begin{tabular}{ll}
\hline
Primer & Locus (direction)\textsuperscript{a} & Nucleotide sequence (5' to 3') \\
\hline
INT1 & int (+) & GCTGGATAAGTTAAGGGCGG \\
INT2 & int (+) & CTCTATGGGCACTGTCCACATTG \\
FLOR-F & floR (+) & TTAATCCCTCTGTTGCTCAGGCG \\
FLOR-B & floR (-) & TCGTCAACTCTGCAAATG \\
SUL2-F & sulI (-) & AAGGGGCGATAGTGTGACG \\
SUL2-B & sulI (+) & TTATCTCCCTGTCGTTCCAGCG \\
STRA-F & strA (+) & CTCTATGGGCACTGTCCACATTG \\
STRA-B & strA (-) & TTATCTCCCTGTCGTTCCAGCG \\
TMP-F & dfrA1 (+) & TGTGCGGATGAAGTCAGCTCC \\
TMP-B & dfrA1 (-) & AGGGGGCGATAGTGTGACG \\
TMP3 & orfA (+) & TTATCTCCCTGTCGTTCCAGCG \\
TMP4 & orfB (-) & CTCTATGGGCACTGTCCACATTG \\
LEN4 & orf1 (+) & CCGTGGATTACACATCTGCC \\
LEFT7 & rumB (-) & GGTGCCATCTCCTCCAAAGTGC \\
RUMA & Intergenic & CATGCTGTTTCTCGCAATGGCG \\
YLF6 & orf73 (+) & CCGTGGATTACACATCTGCC \\
YLF3 & orfC5A (+) & GGGTTGGTTTGGGTTACACATCTGCC \\
\hline
\textsuperscript{a} +, oligonucleotides corresponding to the coding strand (forward primer); -, oligonucleotides corresponding to the noncoding strand (backward primer).
\end{tabular}
\end{table}
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 SXTMO10 as well (Fig. 1). In SXTMO10, floR is found close to the 3' end of the S' -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 phage type DT104 (4) and in an R-plasmid derived from the fish pathogen *Photobacterium damsela* subsp. *piscida* (22). As expected, in-frame deletion of floR from SXTMO10 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 SXTMO10, 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. damsela* (Fig. 1). The genes strA, strB, and sulII, which follow orfA /H11032, are identical to previously described resistance genes and are found on several plasmids, including RSF1010 (35). They encode a sulfonamide-resistant dihydropterate synthase (sulII) and an aminoglycoside phosphotransferase (strAB).

**FIG. 1.** Organization of the antibiotic resistance gene cluster in SXTMO10. The SXTMO10 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 orfA and numB are in black. The numAB operon of R391 is presented above the SXTMO10 antibiotic resistance gene region. The sequence in numB which is repeated in SXTMO10 is in bold and underlined; the flanking imperfect repeat (IR) sequences in SXTMO10 are marked by arrows. Also indicated are the EcoRI sites (E) used for construction of pATMP1 and pSUL1. Regions of nucleotide sequence identity to other published nucleotide sequences are represented by boxes.
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 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 intSXT, a signature sequence of an SXT-related constit, was successful (Table 4). PCR assays utilizing primers that flank the antibiotic resistance genes in SXTMO10 facilitated the mapping of the borders of the DNA missing in strains 2055 and HKO139-SXT. Using chromosomal DNA from either strain as the template for PCR, 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 SXTMO10 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 consins represent a precursor of SXTMO10. Since the num 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 intSXT-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 consin 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 consin designated SXTET, that is very similar but not identical to SXTMO10 (21, 44; data not shown). Using chromosomal DNA from these strains as templates for PCR, they were able to amplify floR, strA, and sulII (Table 4). We will now examine the SXTET in these strains.

### Table 3. Gene products with sequence similarity to the antibiotic resistance gene cluster in SXTMO10

<table>
<thead>
<tr>
<th>Coding region&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Gene name</th>
<th>Length (aa)</th>
<th>Closest similarity</th>
<th>Accession no.</th>
<th>% Identity/range&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–1044 (–)</td>
<td>rum&lt;sup&gt;B&lt;/sup&gt;</td>
<td>197</td>
<td>RumB R391 (C terminus)</td>
<td>862633</td>
<td>39/188 of 530</td>
</tr>
<tr>
<td>1457–2047</td>
<td>orf1</td>
<td>966</td>
<td>Transposable, P. putida</td>
<td>4754812</td>
<td>29/956</td>
</tr>
<tr>
<td>2164–5061</td>
<td>orf2 (ampA)</td>
<td>497</td>
<td>Putative transposable, E. coli</td>
<td>10312101</td>
<td>99/496</td>
</tr>
<tr>
<td>5559–7049</td>
<td>sdrA</td>
<td>184</td>
<td>Dihydrofolate reductase type VIII</td>
<td>28334957</td>
<td>37/157</td>
</tr>
<tr>
<td>7416–7967 (–)</td>
<td>dfr18</td>
<td>181</td>
<td>No homology</td>
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<td>NA</td>
</tr>
<tr>
<td>7984–8526 (–)</td>
<td>orf3</td>
<td>388</td>
<td>No homology</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>8529–9022 (–)</td>
<td>orf4</td>
<td>294</td>
<td>Deyocyclidine triphosphate deaminase, P. aeruginosa</td>
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<td>44/208</td>
</tr>
<tr>
<td>9710–10435 (–)</td>
<td>orf5</td>
<td>110</td>
<td>Putative transposable, E. coli (3’ end, aa 388–497)</td>
<td>10312101</td>
<td>100/278</td>
</tr>
<tr>
<td>11058–11939</td>
<td>orf6</td>
<td>294</td>
<td>No homology</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>12159–13370</td>
<td>floR</td>
<td>404</td>
<td>FloR (floriferol exporter), E. coli</td>
<td>10312100</td>
<td>99/404</td>
</tr>
<tr>
<td>13401–13703</td>
<td>orf7</td>
<td>101</td>
<td>Putative transcriptional regulator (LysR family), P. aeruginosa</td>
<td>11352171</td>
<td>57/71</td>
</tr>
<tr>
<td>13818–14254</td>
<td>orfA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>278</td>
<td>Aminoglycoside phosphotransferase</td>
<td>10312101</td>
<td>100/278</td>
</tr>
<tr>
<td>14332–15165 (–)</td>
<td>strB</td>
<td>267</td>
<td>Aminoglycoside phosphotransferase</td>
<td>1029604</td>
<td>100/267</td>
</tr>
<tr>
<td>15168–15968 (–)</td>
<td>strA</td>
<td>278</td>
<td>Aminoglycoside phosphotransferase</td>
<td>1029604</td>
<td>100/267</td>
</tr>
<tr>
<td>16032–16844 (–)</td>
<td>sulII</td>
<td>272</td>
<td>Dihydropyridate synthase transposable, P. putida (5’ end)</td>
<td>1075456</td>
<td>100/271</td>
</tr>
<tr>
<td>17315–18674</td>
<td>orf2&lt;sup&gt;c&lt;/sup&gt; (ampA&lt;sup&gt;c&lt;/sup&gt;)</td>
<td>240</td>
<td>MutI, V. cholerae</td>
<td>127554</td>
<td>51/173</td>
</tr>
<tr>
<td>18399–19118 (–)</td>
<td>orfB</td>
<td>149</td>
<td>RumA R391 (N terminus)</td>
<td>862632</td>
<td>(217–345 of 563)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Genes encoded on the minus strand are indicated with (–).

<sup>b</sup> 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.

<sup>c</sup> NA, not applicable.
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 SXTET is identical to that in SXTMO10 (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 SXTMO10 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 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 SXTMO10 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-resistant strains, CO943 and 1811/98, as well as in the constin in the Tm-resistant O139 strain AS207 discussed above. We have no direct evidence of the mechanism by which these additional genes were acquired by SXTMO10 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 SXTET, 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. 2. Organization of the region containing antibiotic resistance genes in SXTMO10 and in V. cholerae O139 strains sensitive to Tm, Su, and Cm. The gene order found in strains 2055 and HKO139-SXT (bottom) is compared to that of SXTMO10 (top). Homologous recombination between the identical sequences in orf2 and orf2/HzIII 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.

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 orf4 (top). In contrast, in SXTMO10, 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.
The 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 Tn7. The second closest relative of IntI9 is SpuIntA, the Shewanella putrefaciens chromosomal 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 be located in the cytoplasmatic membrane. 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 SXTET 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 SXTMO10. We took advantage of the partially completed sequence to determine where the dfrA1-containing integron is located in the C10488 consin. Downstream of intI9 in the insert of pYL1 was a region with near nucleotide sequence identity to SXTMO10 (Fig. 4). In SXTMO10, 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 SXTMO10 (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 SXTMO10 (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 SXTMO10, an insert of 4.77 kbp is present in SXTET between traF and an ORF of unknown function, orf73 (Fig. 4). Examination of the borders of the integron in SXTET 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 SXTMO10 and SXTET 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 SXTET 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 nontoxicigenic O139 isolate, E712, that also contained this insertion (Table 4). In fact, like SXTET, the E712 constin also lacked the 3.34-kbp region containing...
**Antimicrob. Agents Chemother.**

**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*', *Sm*', and *Cm*' constin (such as was found in O139 strain AS207) could have become Tol' by acquiring either the novel integron containing *dfrA1*, to give rise to SXT\textsuperscript{ET}, or *dfr18*, *orf3*, *orf4*, and *orf5*, to give rise to SXT\textsuperscript{MO10}. This latter event likely depended on *orfA* (by an unknown mechanism), since *orfA* is associated with antibotic resistance genes in several instances. Subsequently, the *Su*’, *Sm*’, and *Cm*’ 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\textsuperscript{S}. Even though SXT\textsuperscript{MO10} was the first SXT-family constin that we identified (from a 1992 O139 isolate) and we did not detect SXT\textsuperscript{ET} in O1 strains until 1994, given the differences in the antibiotic resistance genes between these two constins, it seems unlikely that SXT\textsuperscript{MO10} is an immediate precursor of SXT\textsuperscript{ET}. Rather, SXT\textsuperscript{ET}, the constin found in most recent O1 isolates, seems to have arisen independently of SXT\textsuperscript{MO10}. We detected an SXT\textsuperscript{ET}-like element in an O139 isolate (E712), indicating that SXT\textsuperscript{ET} is not limited to the *V. cholerae* O1 serogroup. Additionally, we found SXT\textsuperscript{ET} (or at least very similar ele-
ments) in *Providence alcalifaciens* isolates from patients in Bangladesh (data not shown). This suggests a recent gene transfer between *V. cholerae* and *P. alcalifaciens*. Finally, although STX family consins 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 STX family consins to Asian *V. cholerae* remains to be determined.

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