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Structural and Functional Characterization of IS*1358* from *Vibrio cholerae*

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The new epidemic serovar O139 of *Vibrio cholerae* has emerged from the pandemic serovar O1 biotype El Tor through the replacement of a 22-kbp DNA region by a 40-kbp O139-specific DNA fragment. This O139-specific DNA fragment contains an insertion sequence that was described previously (U. H. Stroeher, K. E. Jedani, B. K. Dredge, R. Morona, M. H. Brown, L. E. Karageorgos, J. M. Albert, and P. A. Manning, Proc. Natl. Acad. Sci. USA 92:10374–10378, 1995) and designated IS*1358*$_{O139}$. We studied the distribution of the IS*1358* element in strains from various serovars by Southern analysis. Its presence was detected in strains from serovars O1, O2, O22, O139, and O155 but not in strains from serovars O15, O39, and O141. Furthermore, IS*1358* was present in multiple copies in strains from serovars O2, O22, and O155. We cloned and sequenced four copies of IS*1358* from *V. cholerae* O22 and one copy from *V. cholerae* O155. A comparison of their nucleotide sequences with those of O1 and O139 showed that they were almost identical. We constructed a transposon consisting of a kanamycin resistance gene flanked by two directly oriented copies of IS*1358* to study the functionality of this element. Transposition of this element from a nonmobilizable plasmid onto the conjugative plasmid pOX38-Gen was detected in an *Escherichia coli recA* donor at a frequency of 1.2×10^{-8}. Sequence analysis revealed that IS*1358* duplicates 10 bp at its insertion site.

A new epidemic serovar of *Vibrio cholerae*, designated O139, has recently emerged in India and Bangladesh, where it has been responsible for a large outbreak of cholera (2, 10, 31). The strain from this serovar was the first highly contagious non-O1 strain of *V. cholerae* ever described. It expresses most of the *V. cholerae* O1 virulence factors (1), and further genetic analyses have shown that it probably arose from the pandemic strain of *V. cholerae* O1 biotype El Tor (4, 18, 23, 41). However, in contrast to serovar O1 strains, and like most non-O1 strains, this strain was capsulated and the chemical composition of its lipopolysaccharide (LPS) was different from that of O1 strains (5, 6, 11, 12, 23, 40, 43). Genetic analysis of the region involved in O-antigen biosynthesis, formerly designated the *rfb* locus, has shown that a 22-kb DNA fragment present in O1 strains has been replaced in *V. cholerae* O139 by a 40-kb DNA fragment constituted by (i) seven genes, *wbfA* to *-F* and *wzz*, some of which are likely involved in the regulation of the O-antigen length (*wzz* = *otnB*) and in the capsule transport (*wbtF* = *otnA*) (6, 30, 36); (ii) a putative insertion sequence designated IS*1358* (35); and (iii) 21 open reading frames (ORFs) thought to be involved in O-antigen and capsule biosynthesis (6, 11, 37).

We previously sequenced IS*1358* from *V. cholerae* O139 strain MO45 (ATCC 51394) (GenBank accession no. U24571), which was identical to IS*1358* from O139 strain AI1837 described by Stroeher et al. (35). These 1,326-bp-long insertion sequence (IS) elements have short, nearly perfect (16- or 17-bp) inverted repeats at their ends and encode a putative protein of 375 amino acid (aa) displaying 49% identity with the *Hin*c repeat (H-rpt)-associated protein of the *RhsB* and *RhsE* (rearrangement hot spot) elements found in *Escherichia coli* K-12 strains, 28% identity with the IS*AS1* transposase of *Aeromonas salmonicida* (21), and 31% identity with the PGIS2 transposase of *Porphyromonas gingivalis* (42). A variant of IS*1358* differing by 17 mutations has been described for O1 strains (35). Two of these mutations have generated in-frame stop codons in the IS*1358* transposase gene, leading to the formation of three ORFs, designated *rfbQ*, *rfbR*, and *rfbS*.

The origin of the exogenous DNA in *V. cholerae* O139 is unknown, but this DNA could originate from a non-O1 strain of *V. cholerae*. Consistently, the *wbfA* to *-F* and *wzz* genes have been previously detected in *V. cholerae* strains from serovars O69 and O141 (6), and we have demonstrated that the genes *wbfA* to *-B* are present in strains from serovars O22, O141, and O155 (39). It has been therefore suggested that IS*1358* might be involved in the chromosomal rearrangements that have led to the emergence of serovar O139 from serovar O1, although evidence for transposition of this element is still lacking.

In this work, we studied the distribution of IS*1358* in *V. cholerae* strains from various serovars. We characterized several copies of this IS in O22 and O155 strains which possess O-antigen factors in common with strains from serovar O139 (34), and we demonstrated the functionality of one element originating in a strain from serovar O22.

MATERIALS AND METHODS

Bacterial strains, vectors, and culture media. The *V. cholerae* strains used in this study are listed in Table 1. *E. coli* DH5α (22) and plasmids pUC18 (45) and pSU2718 (26) were used for cloning experiments. *E. coli* HB101 (8) and LC916 (9) and the conjugative plasmid pOX38-Gen (24) were used in the mating assay. DNA fragments to be sequenced were transfected into *E. coli* JM105 (45) by using bacteriophages M13mp18 and M13mp19 (29). All strains were cultured on tryptic soy (TS) broth or agar medium (Difco Laboratories, Detroit, Mich.), except for LC916, which was cultured on brain heart infusion broth or agar medium (Difco). The antibiotics and concentrations used for bacterial selection were as follows: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; rifampin, 100 μg/ml; gentamicin, 5 μg/ml; and streptomycin, 500 μg/ml.

Molecular cloning techniques. Extraction of genomic DNA (17) and small-scale isolation of plasmid DNA (3) were done as described previously. Large-scale plasmid DNA preparations were purified on Qiagen columns in accordance with the manufacturer's recommendations (Qiagen GmbH). Genomic or plasmid DNA was digested with the appropriate restriction endonuclease, and the

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TABLE 1. *V. cholerae* strains used in this study

Strain	Other designation	Serovar	Collection source
N18[a]		O1	J.-M. Fournier
N212		O2	J.-M. Fournier
N226		O15	J.-M. Fournier
N244	169-68	O22	T. Shimada
N217		O39	J.-M. Fournier
N294	1861-79	O69	T. Shimada
N237	MO45 (ATCC 51394)	O139	Y. Takeda
N295	234-93	O141	T. Shimada
N296	490-93	O155	T. Shimada

[a] This strain was isolated during the first wave of the seventh pandemic in Peru in 1991.

resulting fragments were separated by electrophoresis on 0.8% agarose gels and transferred to positively charged nylon membranes (Boehringer, Mannheim, Germany). Prehybridization and hybridization under stringent conditions were carried out as described by the manufacturer (Boehringer). The probe used in this study was a 828-bp DNA fragment internal to IS*1358* from strain MO45 of serovar O139 (IS1358_{O139}) labeled by random priming with 11-dUTP-digoxigenin (Boehringer) or with [α-^{32}P]dCTP. This fragment, designated *rfbQRS*$_{O139}$, was amplified by PCR from *V. cholerae* O139 strain MO45 by using the primer set 5′-ACTGACGGATGGTGAA-3′ and 5′-TCACGTAAGGCTTTCAAGAA-3′. The PCR was performed as follows. Fifty nanograms of target DNA, 200 mM each deoxynucleoside triphosphate, 0.1 nmol of each primer, and 1 U of thermostable DNA polymerase (New England Biolabs, Beverly, Mass.) were mixed in the corresponding 1X polymerase buffer. Amplification involved 35 cycles, each consisting of (i) a denaturation step of 1 min at 94°C, (ii) an annealing step of 1 min at 55°C, and (iii) a polymerization step of 1 min 30 at 72°C. The resulting amplicon was purified from agarose gels by use of the Geneclean kit (Bio 101, La Jolla, Calif.) before labeling was performed.

Pulsed-field gel electrophoresis (PFGE). Extraction of bacterial DNA from *V. cholerae* strains grown for 18 h at 37°C was performed as described previously (27). Total DNA was digested by *Sfi*I (30 IU), and the resulting fragments were separated by electrophoresis on a 1.0% agarose gel (150 V for 28 h with total pulse times of 7 to 28 s) by use of a contour-clamped homogeneous-field electrophoresis apparatus (CHEF-DR II; Bio-Rad, Richmond, Calif.).

RNA isolation and dot blot analysis. Total RNA was extracted from exponentially growing *V. cholerae* strains (10 ml of a bacterial culture with an optical density at 600 nm between 0.8 and 0.9) that had been cultured in TS broth as previously described (7). Equal amounts of RNA (10 μg) were then denatured for 15 min at 65°C in the presence of 7% formaldehyde. The sample volume was brought to 200 μl to facilitate the filling of the wells in the slot blot apparatus (Bio-Rad).

Sequencing of IS*1358* from O22 and O155 strains. There is no *Hin*cII restriction site in IS1358_{O139}. Therefore, in order to clone related IS elements in *V. cholerae* O22, *Hin*cII-restricted DNA from strain N244 was separated by electrophoresis through an 0.8% agarose gel, and 1.4- to 3-kb DNA fragments were extracted from a low-melting-point agar gel and ligated with T4 DNA ligase into *Sma*I-digested pUC18 vector (Appligene, Illkirch, France). Recombinant plasmids were introduced into *E. coli* DH5α by transformation, and transformants were selected on TS agar containing ampicillin and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). White transformants resistant to ampicillin were screened for the presence of the IS*1358*-related sequences by colony blot hybridization (32) with the specific probe. Four clones gave positive signals, and restriction analysis (*Eco*RI or *Hin*dIII) of their plasmid content revealed that each recombinant molecule carried a different insert. These inserts were subsequently cloned into the replicative forms of M13 mp18 and M13 mp19 phages. The sequences of all four copies of IS*1358* and their flanking regions were determined on both DNA strands by the dideoxynucleotide chain termination method (33) using modified T7 DNA polymerase (Sequenase version 2.0; Amersham, France) and primers derived from the known sequence of IS1358_{O139} (35) and from the sequence determined in this work. By using the same approach, we characterized one copy of IS*1358* from *V. cholerae* O155 (strain N296).

Construction of an IS*1358*-based transposon. The pairs of primers KpnQRS2 (5′-CGGGGTACCGACAGCTAAACGAGCAATGCAGGG-3′) and BamSRQ (5′-CGCGGATCCATTGATTTGAAAGCCTTGCCCGACA-3′) or BamQRS2 (5′-CGCGGATCCGACAGCTAAACGAGCAATGCAGGG) and PstSRQ (5′-AAACTGCAGATTGATTTGAAAGCCTTGCCCGACA-3′) were used to amplify a 1,420-bp DNA fragment containing IS$1358_{O22\text{-}3}$ (copy 3 of IS*1358* from *V. cholerae* O22) plus 19 and 76 bp of the upstream and downstream flanking regions, respectively (the polarity of the element being arbitrarily defined as the direction of transcription of the transposon-encoded transposase). These primers were designed to generate copies of IS$1358_{O22\text{-}3}$ flanked by *Kpn*I and *Bam*HI or *Bam*HI and *Pst*I sites (underlined bases), which were designated IS$1358_{O22\text{-}3R}$ (right end) and IS$1358_{O22\text{-}3L}$ (left end), respectively. The PWO (*Pyrococcus wosei*) DNA polymerase (Boehringer) was used to minimize the misincorporation of nucleotides during PCR, and sequencing of one strand of the amplified IS$1358_{O22\text{-}3L}$ did not reveal any mutation. These two amplified ISs, after digestion with the appropriate enzymes, were mixed with a 1.5-kb *Bam*HI fragment containing the kanamycin resistance gene *aphA-3* (38) and with plasmid pSU2718 digested with *Kpn*I and *Pst*I and then treated with T4 DNA ligase, and the ligation products were introduced by transformation into *E. coli* DH5α. Restriction analysis (with *Eco*RI and *Hin*dIII) of the plasmid content of clones resistant to ampicillin and kanamycin revealed the presence in all eight clones studied of a pSU2718 derivative harboring IS$1358_{O22\text{-}3R}$ and IS$1358_{O22\text{-}3L}$, in direct orientation, separated by the *aphA-3* gene. This composite transposon constructed in vitro was designated Tn*1358*-Km. We also constructed pSU2718ΩKm, an IS-free pSU2718 derivative containing only the kanamycin resistance gene *aphA-3*.

Mating assay. The transposition and cointegrate-forming properties of IS*1358* and Tn*1358*-Km were studied in a mating assay as described previously (19). In this system, the mobility of a transposable element carried by a nontransferable and nonmobilizable plasmid to a self-transferable plasmid was revealed in a standard mating assay between the *recA* strains *E. coli* LC916, used as a donor, and *E. coli* HB101, used as a recipient. Plasmid pOX38-Gen, a conjugative F derivative which does not carry any known insertion elements except a small region of IS*3*, was used as a target molecule. The nonmobilizable plasmids pSU2718 and pUC18-Km, a pUC18 derivative in which the *bla* gene was replaced by the kanamycin resistance gene *aphA-3*, were used as transposon delivery vectors. Plasmids pUC18-KmΩIS*1358* and pSU2718ΩTn*1358*-Km were used to detect the formation of cointegrates (pOX38-Gen::pUC18-KmΩIS*1358* and pOX38-Gen::pSU2718ΩTn*1358*-Km), whereas only the latter replicon was used to characterize the direct transposition events (pOX38-Gen::Tn*1358*-Km). In these experiments, pUC18-Km and pSU2718ΩKm, a derivative of pSU2718 carrying the *aphA-3* gene, were used to determine the background level of mobilization.

Nucleotide sequencing of the transposon target junctions in pOX38-Gen. Genomic DNA of a transconjugant resulting from a direct transposition event was digested with *Taq*I and self-ligated. A PCR was then performed with the primer pair 5′-AGCCTTACGTGACGGTGATGTTCAT-3′ and 5′-GGTACTT TTCGTCCATTGCGCAG-3′ to characterize IS1358_R::pOX38-Gen junction sequences. The amplified DNA fragment was then cloned into pUC18 and sequenced. Sequence analysis was performed to determine the exact insertion site of Tn*1358*-Km in pOX38-Gen. A second PCR was performed with primers 5′-GCGGCAAGTACGGCACTCAGACGG-3′ and 5′-CACCGCAGCCCTTA TATATCAACGA-3′, and the resulting 293-bp fragment corresponding to the IS1358_L::pOX38-Gen junction fragment was sequenced.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper will appear in the GenBank nucleotide sequence database under accession no. AF004381 to AF004383 and AF004385 for strain N244 of *V. cholerae* O22 and under accession no. AF004384 for strain N296 of *V. cholerae* O155.

RESULTS

Distribution of the IS*1358* element in various serovars of *V. cholerae*. We studied, by Southern blot analysis, the distribution of the IS*1358* element among a selection of wild-type strains from various serovars of *V. cholerae*. The *Sfi*I-restricted chromosomal DNA fragments of strains from serovars O1, O2, O15, O22, O39, O139, O141, and O155 were separated by PFGE and hybridized under high-stringency conditions with the *rfbQRS*$_{O139}$ probe. As illustrated in Fig. 1, hybridizing bands were detected only with DNA from strains from serovars O1, O2, O22, O139, and O155. Furthermore, IS*1358* homologous sequences were present in multiple copies in strains from serovars O2, O22, and O155 whereas a single copy was found in strains from *V. cholerae* serovars O1 and O139. Interestingly, IS*1358* was not detected in strains from serovars O15, O39, and O141. There is no *Sfi*I site within the IS*1358* element of *V. cholerae* O139, and we therefore estimated the IS copy number in strains from serovars O2, O22, and O155 as the number of bands hybridizing with the probe: four copies were detected in *V. cholerae* O2 strains, four copies were detected in *V. cholerae* O22 strains, and six copies were detected in *V. cholerae* O155 strains. This constitutes a rough estimation of the copy number since several copies might be present in the same band, a feature which would lead to an underestimation, and/or some copies might contain an internal *Sfi*I site, which would lead to an overestimation. Multiple copies of IS*1358*

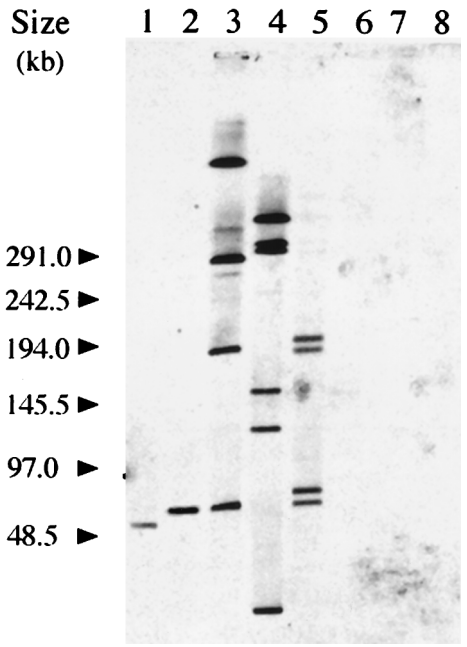

FIG. 1. Southern blot analysis of *V. cholerae* genomic DNAs. *Sfi*I-digested DNAs were separated by PFGE, transferred to a nylon membrane, and hybridized with a digoxigenin-labeled DNA probe specific for IS*1358*$_{O139}$. Bacterial strains (serovars) were N18 (O1), MO45 (O139), N244 (O22), N296 (O155), N212 (O2), N295 (O141), N217 (O39), and N226 (O15) (lanes 1 to 8, respectively). Bacteriophage lambda concatemers were used as molecular size markers.

(four or more) were also found in strain N294 (serovar O69) (data not shown).

Transcriptional analysis of IS*1358* elements in various *V. cholerae* strains. A slot blot analysis was performed on RNAs extracted from exponentially growing cultures by using an *rfbQRS*$_{O139}$-specific DNA probe. As shown in Fig. 2, transcripts corresponding to IS*1358* were detected in all strains harboring this element. The intensity of the hybridizing dots was significantly higher in strains from two (O22 and O69) of the four serovars containing multiple copies of IS*1358* (O2, O22, O69, and O155). In these experiments, strains from the IS*1358*-free serovars O15 and O141 were used as negative controls (Fig. 2). These results might suggest that in strains

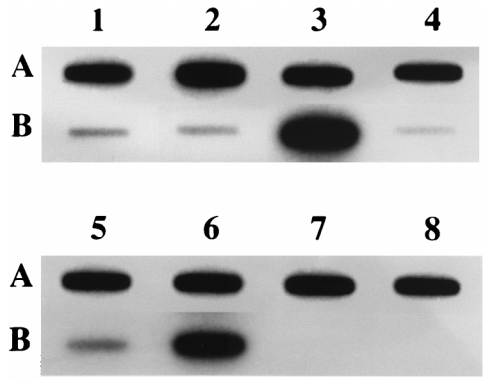

FIG. 2. Slot blot analysis of IS*1358* transcription in various *V. cholerae* strains. Total RNAs (5 μg) were spotted onto a nylon membrane and hybridized with ^{32}P-labeled DNA probes specific for IS*1358*$_{O139}$ (B) or for *V. cholerae* 16S rDNA (A). Bacterial strains (serovars) were N18 (O1), MO45 (O139), N244 (O22), N296 (O155), N212 (O2), N294 (O69), N226 (O15), and N295 (O141) (lanes 1 to 8, respectively).

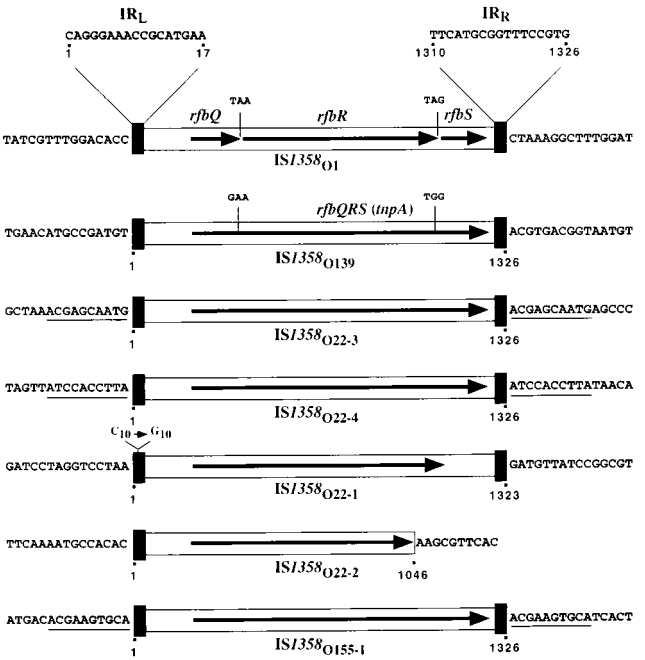

FIG. 3. Schematic comparison of IS*1358* elements originating from various *V. cholerae* strains. IS*1358*$_{O1}$, IS*1358*$_{O139}$, and IS*1358*$_{O155}$ originate from strain (serovar) N18 (O1), MO45 (O139), and N296 (O155), respectively; the four copies of IS*1358*$_{O22}$ originate from strain N244 (O22). The prototype sequence IS*1358*$_{O1}$ contains three ORFs, designated *rfbQ*, *rfbR*, and *rfbS*, which, due to point mutations, were fused in a single ORF designated *rfbQRS* (or *tnpA*) in IS*1358*$_{O139}$, IS*1358*$_{O22-3}$, IS*1358*$_{O22-4}$, and IS*1358*$_{O155-1}$. IS*1358*$_{O22-1}$ contains a truncated *tnpA* gene due to the presence of an in-frame stop codon. IS*1358*$_{O22-2}$ is a 270-bp deletion derivative devoid of the IR$_R$. The six ISs of similar size display a high level of sequence identity (≥93%). Symbols: heavy black boxes represent the 17-bp IR$_L$ and IR$_R$; horizontal arrows delineate the direction of transcription and extent of *tnpA*. The sequences of IR$_L$ and IR$_R$ and of the target sites are shown. The 10-bp sequences duplicated at the insertion sites of IS*1358*$_{O22-3}$ and IS*1358*$_{O22-4}$ are underlined.

from serovars O22 and O69, at least one copy of IS*1358* is transcribed from a strong chromosome-borne promoter.

Sequence analysis of IS*1358* elements from various *V. cholerae* serovars. We cloned and sequenced four copies of IS*1358* from *V. cholerae* O22 (designated IS*1358*$_{O22-1}$, IS*1358*$_{O22-2}$, IS*1358*$_{O22-3}$, and IS*1358*$_{O22-4}$) and one copy from O155 (designated IS*1358*$_{O155-1}$). Sequence analysis revealed that IS*1358*$_{O22-3}$, IS*1358*$_{O22-4}$, and IS*1358*$_{O155-1}$ were almost identical to IS*1358*$_{O139}$. They had an identical size of 1,326 bp, displayed greater than 96% sequence identity, possessed identical 17-bp inverted repeats at their extremities, and, unlike IS*1358*$_{O1}$, contained a single ORF coding for highly homologous 375-aa putative proteins (≥98% identity). This ORF was preceded by a putative ribosome binding site (GGAGC) located 6 bp upstream from the ATG start codon. Interestingly, IS*1358*$_{O22-3}$, IS*1358*$_{O22-4}$, and IS*1358*$_{O155-1}$ were flanked by 10-bp direct repeats (Fig. 3). The 1,326-bp IS*1358*$_{O22-1}$ is also highly homologous to IS*1358*$_{O139}$ (97% identity), but its left inverted repeat (IR$_L$) contained a C-to-G mutation at position 10. However, due to a mutation generating an in-frame stop codon, this IS coded for a 329-aa putative transposase truncated at its carboxylic moiety. IS*1358*$_{O22-4}$ was a truncated form of the IS*1358* that had lost 270 bp of the segment containing the 3′ moiety of the putative transposase gene and the inverted repeat designated the right inverted repeat (IR$_R$) (Fig. 3). Sequence analysis revealed that (i) IS*1358*$_{O1}$ is inserted into a noncoding region located between *rfbO* and *rfbT* of *V. cholerae* O1; (ii) IS*1358*$_{O139}$ and IS*1358*$_{O22-1}$ are inserted

TABLE 2. Conjugative transfer of resistance determinants from *E. coli* LC916 to *E. coli* HB101

Plasmid content of the donor	Antibiotics used for selection[a] and concn (μg/ml)	Transfer frequency[b]
pOX38-Gen + pUC18-KmΩIS*1358*	SM, 500; GM, 5	0.8
	SM, 500; KM, 50	1.1×10^{-7}
pOX38-Gen + pUC18-Km	SM, 500; GM, 5	0.8
	SM, 500; KM, 50	2.3×10^{-7}
pOX38-Gen + pSU2718ΩTn*1358*-Km	SM, 500; GM, 5	0.6
	SM, 500; KM, 50	5.9×10^{-6}
pOX38-Gen + pSU2718ΩKm	SM, 500; GM, 5	0.7
	SM, 500; KM, 50	7.8×10^{-7}

[a] SM, streptomycin; KM, kanamycin; GM, gentamicin.
[b] Transfer frequencies were expressed as the number of transconjugants per donor CFU after mating.

at the 3′ ends of the *wzz* gene of *V. cholerae* O139 and *V. cholerae* O22, respectively (16); and (iii) IS*1358*$_{O22\text{-}4}$ is inserted at the 5′ extremity of a 59-bp element belonging to a novel class of integron recently described for the *V. cholerae* O1 genome (28). The sequences of the segments flanking IS*1358*$_{O1}$, IS*1358*$_{O139}$, IS*1358*$_{O22\text{-}1}$, IS*1358*$_{O22\text{-}2}$, IS*1358*$_{O22\text{-}3}$, IS*1358*$_{O22\text{-}4}$, and IS*1358*$_{O155\text{-}1}$ were structurally unrelated.

IS*1358* mediates direct transposition. The ability of IS*1358* to mediate cointegrate formation was studied in a mating assay by using an *E. coli recA* donor harboring either pOX38-Gen plus pUC18-KmΩIS*1358* or pOX38-Gen plus pUC18-Km (Table 2). In these experiments, transfer of the Km^r determinant of pUC18-KmΩIS*1358* and of pUC18-Km was detected at frequencies of 1.1×10^{-7} and of 2.3×10^{-7}, respectively. The plasmid content of seven clones harboring pOX38-Gen::pUC18-KmΩIS*1358* cointegrates originating from the same experiment and corresponding to all transconjugants obtained at the penultimate proficient dilution was digested with *Eco*RI and studied by Southern blot analysis with *rfbQRS*$_{O139}$, the IS*1358*-specific DNA probe. This analysis revealed that all pOX38-Gen::pUC18-KmΩIS*1358* cointegrates contained a single copy of the IS element (data not shown). These results suggest that the formation of cointegrates between pOX38-Gen and pUC18-KmΩIS*1358* were not IS mediated and that IS*1358* does not mediate cointegrate formation, at least in an *E. coli* genetic background. The fact that transfer of the IS-free vector pUC18-Km occurred at a frequency similar to that of pUC18-KmΩIS*1358* is consistent with this proposal (Table 2).

The transposon Tn*1358*-Km, in which the kanamycin resistance gene *aphA-3* is flanked by two directly oriented copies of IS*1358*$_{O22\text{-}3}$, was constructed to study the ability of this IS to mediate direct transposition. In mating experiments between LC916 donors harboring pOX38-Gen plus pSU2718ΩTn*1358*-Km and HB101 recipients, transfer of the Km^r determinant was detected at an average frequency of 5.9×10^{-6} (Table 2). The cotransfer of the chloramphenicol resistance marker of the vector pSU2718 was tested on transconjugants harboring Tn*1358*-Km. This analysis revealed that in the three mating experiments performed, the majority (≥80%) of the clones resistant to kanamycin were also resistant to chloramphenicol. We assume that these clones do not result from an IS-mediated cointegration event because the transfer of the IS-free vector pSU2718ΩKm was detected at a similar frequency of 7.8×10^{-7} (Table 2). Moreover, a Southern blot analysis revealed that the hybridization profile of the corresponding cointegrates obtained with the IS*1358* probe is indistinguishable from that of pSU2718ΩTn*1358*-Km (Fig. 4 shows part of this analysis), thus suggesting that there is no IS duplication. The plasmid content of the four Km^r transconjugants harboring Tn*1358*-Km susceptible to chloramphenicol was studied by Southern blot analysis; the study revealed an indistinguishable hybridization pattern and the presence of two copies of IS*1358*, one of which was associated with a novel plasmid-transposon junction fragment (Fig. 5). We also demonstrate that the pOX38-Gen::Tn*1358*-Km molecules were devoid of sequence related to pSU2718 (data not shown). Taken together, these results demonstrate the transposition of Tn*1358*-Km at the same location in pOX38-Gen. Sequence analysis of the Tn*1358*-Km insertion site in pOX38-Gen revealed that transposition occurred within the *traD* gene and resulted in a 10-bp duplication of the target DNA (Fig. 5). Our inability to retransfer pOX38-Gen::Tn*1358*-Km from HB101 to LC916 (data not shown) is thus due to the insertional inactivation of the *traD* gene of pOX38-Gen with Tn*1358*-Km. It is noteworthy that the estimated size (3.7 kb) of the *Eco*RI IS*1358*$_R$-*traD* junction fragment corresponded to that calculated from the nucleotide sequence (Fig. 5).

DISCUSSION

In this work, we studied the distribution of the IS*1358* element among a selection of wild-type *V. cholerae* strains from various serovars. This analysis revealed that sequences related to IS*1358* were present in strains from serovars O1, O2, O22, O139, and O155 but not in strains from serovars O15, O39, and O141. These results suggest that the acquisition of the IS*1358* element by *V. cholerae* is a relatively recent event which occurred after the bacterial speciation. Southern analysis revealed that multiple copies of this element were found in strains O2 (four or more copies), O22 (four or more copies), and O155 (six or more copies) whereas a single copy was detected in strains from serovars O1 and O139. The nucleotide sequences of IS*1358*$_{O1}$ (25) and IS*1358*$_{O139}$ (35) have been previously published. We therefore determined the nucleotide sequences of four copies of IS*1358* from *V. cholerae* O22 (designated IS*1358*$_{O22\text{-}1}$, IS*1358*$_{O22\text{-}2}$, IS*1358*$_{O22\text{-}3}$, and IS*1358*$_{O22\text{-}4}$) and of one copy of IS*1358* from *V. cholerae* O155 (IS*1358*$_{O155\text{-}1}$) to carry out a detailed analysis of the sequence heterogeneity of this element in this species. Sequence analysis revealed that IS*1358*$_{O22\text{-}3}$, IS*1358*$_{O22\text{-}4}$, and IS*1358*$_{O155\text{-}1}$ were almost identical to IS*1358*$_{O139}$ and IS*1358*$_{O1}$. These 1,326-bp elements

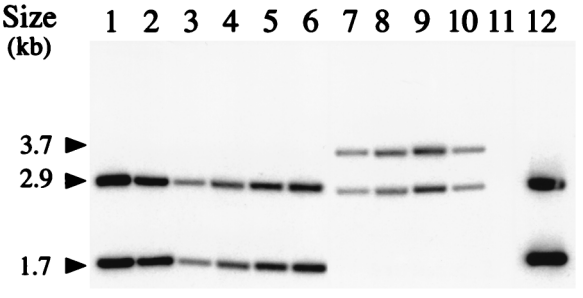

FIG. 4. Southern blot analysis of the transposition behavior of IS*1358*$_{O139}$ in *E. coli*. Genomic DNAs were digested with *Eco*RI, separated in an 0.8% agarose gel, transferred to a nylon membrane, and hybridized with a ^{32}P-labeled DNA probe specific for IS*1358*$_{O139}$. Bacterial strains were chloramphenicol-resistant HB101 transconjugants harboring pOX38-Gen::pSU2718ΩTn*1358*-Km (lanes 1 to 6), chloramphenicol-sensitive HB101 transconjugants harboring pOX38-Gen::pSU2718ΩTn*1358*-Km (lanes 7 to 10), LC916 donor harboring pOX38-Gen plus pSU2718ΩKm (lane 11), and LC916 donor harboring pOX38-Gen plus pSU2718ΩTn*1358*-Km (lane 12). The 1-kb ladder (Gibco-BRL) was used for molecular size markers.

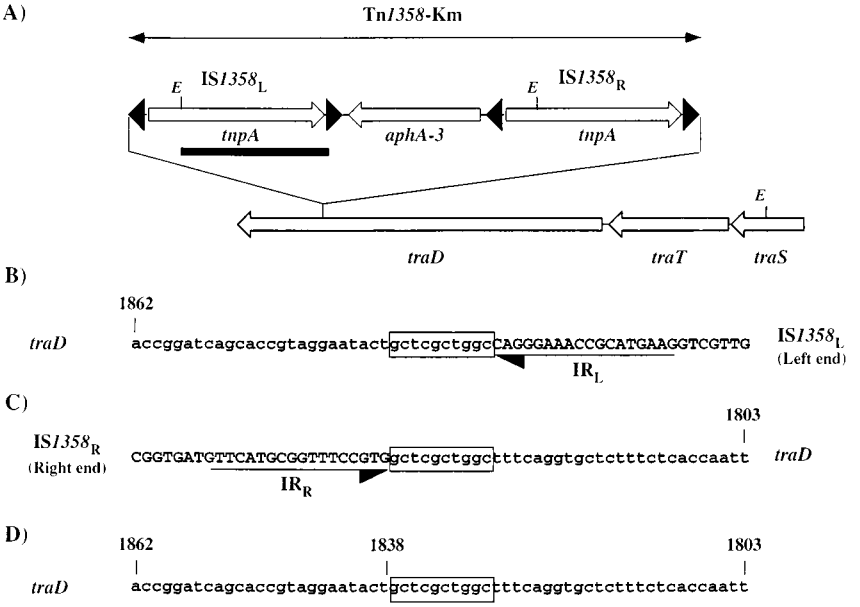

FIG. 5. Insertion of Tn*1358*-Km into pOX38-Gen. (A) The partial restriction map of the *traD* locus of pOX38-Gen::Tn*1358*-Km is shown. The open arrows indicate the directions of transcription of the genes *tnpA*, *aphA-3*, *traD*, *traT*, and *traS*. The closed arrowheads represent the 17-bp left IRs of IS*1358*. The black bar below IS*1358*$_L$ delineates the IS-specific DNA probe. *E*, *Eco*RI site. (B to D) The nucleotide sequences of the left (B) and right (C) pOX38-Gen::Tn*1358*-Km junction fragments and of the corresponding segment of the F *traD* gene (D) are indicated. The sequences of the *traD* gene and of IS*1358* are indicated by lower- and uppercase letters, respectively. The horizontal arrows delineate the 17-bp IR of IS*1358*, and the sequence of the 10-bp duplicate motif at the insertion site is boxed. The coordinates refer to the first base of the *traD* gene (GenBank accession no. M29254).

displayed more than 96% nucleotide identity, possessed identical 17-bp inverted repeats at their extremities, and, with the exception of IS*1358*$_{O1}$, contained a single ORF coding for highly homologous 375-aa putative proteins (≥98% of identity). This putative TnpA is homologous to the H-rpt-associated protein of *RhsB* and *RhsE* found in *E. coli* K-12 (49% identity) and to the H-rpt elements associated with loci that determine O-antigen biosynthesis genes in *Salmonella enterica* (44). It also displays 28% identity with the IS*AS1* transposase of *A. salmonicida* (21) and 31% identity with the PGIS2 transposase of *P. gingivalis* (42). IS*1358*$_{O22-1}$ is a 1,326-bp element containing a truncated TnpA due to the presence of an in-frame stop codon. IS*1358*$_{O22-2}$ is a 270-bp deletion derivative of IS*1358* which does not contain the right inverted repeat. Interestingly, IS*1358*$_{O22-3}$, IS*1358*$_{O22-4}$, and IS*1358*$_{O155}$ were flanked by 10-bp direct repeats, a feature which might indicate that the corresponding IS elements are functional.

To study the functionality of IS*1358*, we constructed a compound transposon, designated Tn*1358*-Km, in which the kanamycin resistance gene *aphA-3* was flanked by two directly oriented copies of IS*1358*$_{O22-3}$. By using a mating assay in an *E. coli* genetic background, we demonstrated that IS*1358*$_{O22-3}$ is able to mediate direct transposition but does not mediate the formation of cointegrates. Insertion of Tn*1358*-Km was obtained at a single locus of pOX38-Gen. Sequence analysis revealed that insertion occurred within the *traD* gene and resulted in a 10-bp duplication of the target DNA. The insertion-inactivation of a gene belonging to the transfer operon accounts for the inability of pOX38-Gen::Tn*1358*-Km to retransfer from HB101 donors to LC916 recipients. The transposition frequency of Tn*1358*-Km onto pOX38-Gen, determined by dividing the frequency of the conjugative transfer of the kanamycin resistance determinant from LC916 to HB101 (5.9×10^{-6}) by (i) the transfer frequency of pOX38-Gen (6×10^{-1}), (ii) the copy number of pSU2718 (20 copies per cell), (iii) the number of generations of the donor cell before selection (about eight generations), and (iv) the percentage of transconjugants originating from a direct transposition event (20%), was 1.2×10^{-8}. This transposition frequency is comparable to those calculated for many other IS elements (20). Sequence analysis of IS*1358* insertion sites associated with a 10-bp target duplication in *V. cholerae* O22 (IS*1358*$_{O22-3}$ and IS*1358*$_{O22-4}$) and O155 (IS*1358*$_{O155-1}$) genomes and in the *traD* gene of pOX38-Gen did not reveal any obvious consensus motif for integration. Finally, it is important to note that the ability of IS*1358* to translocate as a compound transposon might account for the fact that IS*1358*$_{O1}$, IS*1358*$_{O139}$, and IS*1358*$_{O22-1}$ were not flanked by a 10-bp duplication (Fig. 3).

The new epidemic strain from serovar O139 of *V. cholerae* has probably emerged from the pandemic O1 biotype El Tor through a genetic rearrangement involving the horizontal transfer of exogenous O-antigen- and capsule-encoding genes of unknown origin. It has been reported that *V. cholerae* strains from serovars O22 and O155 possess O-antigen factors in common with *V. cholerae* serovar O139 strains (34). Furthermore, structural analysis of the LPS from *V. cholerae* serovars O22 and O139 have recently revealed that strains from these two serovars had almost the same O-antigen repeat unit (13–15). The presence of an IS element within these regions in both serovars O1 and O139 addresses the question of the role of IS*1358* in the horizontal transfer of genes encoding O139 LPS biosynthesis and on the origin of the exogenous DNA. It is generally assumed that the cointegration pathway leads to large genome rearrangements whereas the direct transposition pathway results in the addition of small DNA fragments. Thus, if we assume that the transposition behavior of IS*1358* is similar in *E. coli*, where it only mediates direct transposition, and in *V. cholerae*, it is unlikely that this element is directly implicated in the acquisition of novel O-antigen and capsule biosynthesis genes by *V. cholerae* O139.

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