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Spontaneous Insertion of an IS*1*-Like Element into the *virF* Gene Is Responsible for Avirulence in Opaque Colonial Variants of *Shigella flexneri* 2a

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Colonial variation of *Shigella flexneri* serotype 2a from the translucent (2457T) to the opaque form (2457O) occurs spontaneously once in 10^4 cell divisions, with concomitant loss of *ipa* gene expression and virulence. The appearance of 2457O was associated with the insertional inactivation of *virF*, an invasion plasmid-encoded positive regulator of *ipa* gene expression. Plasmid pWR110, a Tn*5*-tagged invasion plasmid that restores the invasive phenotype to plasmid-cured *Shigella* derivatives, was conjugally transferred into 2457O. Synthesis of the invasion-associated IpaB and IpaC polypeptides, normally present on the surface of virulent shigellae, and the invasive phenotype were restored in 2457O(pWR110) transconjugants. Plasmid DNA restriction endonuclease patterns of 2457T and 2457O, along with hybridization analysis, showed that a *Sal*I fragment carrying the *virF* gene in 2457O had increased in size relative to its counterpart in 2457T. Analysis of *virF* DNA sequences amplified by the polymerase chain reaction revealed that the *virF* sequence from 2457O was 780 bp larger than that amplified from 2457T. Moreover, the *virF* sequence amplified from 2457O hybridized to an IS*1* DNA probe whereas the amplified 2457T *virF* sequence did not. DNA sequence analysis mapped the insertion element, designated IS*1*SFO, within an A · T-rich region of the *virF* open reading frame and identified a 9-bp *virF* target sequence that was duplicated at the insertion site of IS*1*SFO. The DNA sequence of IS*1*SFO was >99% homologous to IS*1*F. Plasmid pWR600, carrying a 1,260-bp *Hpa*II fragment encoding a wild-type *virF* gene, was able to restore the virulent phenotype and translucent colonial morphology to nine independently isolated 2457O hosts.

Shigella species have been extensively studied to define the various steps in the pathogenic mechanism of shigellosis and to determine the complement of *Shigella* genes required for expression of the virulent phenotype. Spontaneous, transposon-induced, and site-directed mutations (22, 45, 47) have provided a variety of *Shigella* mutants defective in particular steps of the infectious process. Characterization and complementation analysis of these mutants, which have lost the capacity to attach and invade, escape from the phagocytic vacuole, replicate intracellularly, or disseminate to adjacent colonic epithelial cells, have established a sequela of events that produce the dysentery syndrome. Molecular examination of the mutants has revealed a complex array of plasmid- and chromosome-encoded virulence determinants. Some loci directly affect the ability of shigellae to invade and survive intracellularly, whereas other loci regulate expression of the virulent phenotype (reviewed in reference 16).

A 1964 study compared the virulence of a *Shigella flexneri* serotype 2a mutant with that of the parental strain (22). The mutant strain, designated 2457O, was isolated on meat extract agar (MEA) as an opaque colonial variant of the translucent colony-forming strain 2457T. Rhesus monkeys orally challenged with 2457T developed diarrheal symptoms and intestinal lesions, whereas 2457O caused neither symptoms nor pathology. Histological examination of the intestinal tissues from challenged monkeys showed that the 2457T cells had invaded and multiplied within the colonic epithelium of the host. In contrast, the 2457O cells were found only in the intestinal lumen. This study established that shigellae cause disease not by elaborating a toxin while adhering to the surface of the intestinal epithelium (57) but rather by penetrating the intestinal mucosa.

Attempts to characterize the underlying genetic alteration that conveys avirulence to the opaque colonial variant have focused on both plasmid and chromosomal loci. Analysis of plasmids from 2457T and 2457O did not reveal any detectable alteration in plasmid content or molecular size, and plasmid DNA restriction patterns generated with *Bam*HI and *Eco*RI enzymes showed no apparent differences between the two cell types (21). In addition to the loss of virulence, one 2457O isolate has been reported to have lost glycerol kinase activity ($GlpK^-$). Transduction experiments, which selected for the transfer of the chromosome-encoded *glpK* gene from 2457T to this 2457O isolate, restored the invasive phenotype to 39% of the recipients, and 50% of the spontaneously isolated 2457O $GlpK^+$ revertants also regained invasiveness (20). More recently, it has been shown that the Ipa polypeptides, which are surface exposed (30) and required for shigellae to invade epithelial cells (45), are not synthesized by 2457O (17). The data from these three studies (17, 20, 21) suggest that a genetic locus on the *Shigella* chromosome, associated with the $GlpK^+$ phenotype, could affect *ipa* gene expression and be the site of a mutation that converts virulent 2457T to avirulent 2457O.

In this study, we have characterized a mutation responsible for the spontaneous appearance of nine independently isolated 2457O variants and demonstrate that this mutation is not associated with the $GlpK^+$ phenotype. All nine 2457O isolates had a unique IS*1*-like element inserted into the open reading frame of the invasion plasmid-encoded *virF* gene.

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Insertional inactivation of the *virF* gene in 2457O blocks expression of the *ipa* genes, rendering the variant noninvasive and consequently avirulent.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. In 1954, *S. flexneri* serotype 2a strain 2457T was isolated from a shigellosis patient by O. Felsenfeld in Tokyo, Japan, and sent to S. Formal at the Walter Reed Army Institute of Research (13). In the same year, Formal isolated a colonial variant of 2457T and designated it 2457O (11). Both of these strains and the 2457O strain that was used in the 1964 monkey challenge study (22) were maintained in a lyophilized state until opened for this study. In addition, seven other 2457O variants were independently isolated in 1990 for this study. A log-phase culture growing in Penassay broth (Difco Laboratories, Detroit, Mich.), inoculated from a single 2457T colony, was plated on MEA (Difco Laboratories), incubated at 37°C, and examined for the spontaneous appearance of the O-type colonies. To ensure that each 2457O isolated was from an independent event and not from the multiplication of a previously generated 2457O, only one 2457O variant was isolated from each screening experiment. Strain 2457T has a green-gold translucent (T) colonial form on MEA when examined under a dissecting microscope by oblique transmitted light. It spontaneously segregates into strain 2457O, a stable orange opaque (O) colonial variant, once in every 10^4 cell divisions (22). *Shigella* strains were routinely tested in an agglutination reaction with *S. flexneri* 2a-specific antiserum prepared by S. Formal.

Virulence assays. *Shigella* strains were screened by techniques previously described for their capacity to bind Congo red dye (26, 36a); synthesize IpaA, IpaB, IpaC, IpaD, and VirG polypeptides (32); lyse sheep erythrocytes (44); invade HeLa cells (10); form plaques in HeLa cell monolayers (33); and elicit a keratoconjunctivitis reaction in guinea pigs (48). In addition, strains were screened for the presence of IpaB and IpaC polypeptides on the cell surface in an immunocolony assay. In this procedure, the bacteria were streaked over the surface of a nitrocellulose filter overlaid on MEA containing the appropriate antibiotic. After overnight incubation at 37°C, the filter was lifted and screened with IpaB- or IpaC-specific monoclonal antibodies as described previously (30).

Mating procedures. *Escherichia coli* K-12 strain 7262-1-10 (395-1 *pro thr leu arg his thi mtl gal lac*) (42), which carries the 220-kb Tn*5*-tagged invasion plasmid of *S. flexneri* 5 strain M90T (43), served as the donor in the mating experiment. 7262-1-10 is an invasive isolate that harbors a cointegrate molecule of the tagged invasion plasmid (pWR110) and the mobilizing plasmid (R64*drd*11) that encodes tetracycline and streptomycin resistance. The transfer of pWR110 to 2457O variants was performed as described by Miller (28), with the following modifications: after allowing an hour for DNA transfer, the mating mix (1:2 ratio of donor to recipient) was diluted 10-fold into fresh Penassay broth and incubated, with shaking, for 1 h at 37°C to allow expression of kanamycin resistance. Cells in the mating mixture were washed twice in saline before being plated on M9 minimal plates (25) supplemented with nicotinic acid (2 μg/ml) to satisfy the growth requirements of the *Shigella* recipient and kanamycin (50 μg/ml) to select for pWR110 transfer. The donor strain is auxotrophic for several amino acids and was counterselected by the absence of these amino acids in the selection plates.

Analysis of plasmid DNA. Large-scale isolation of plasmid DNA was carried out as previously described by Cassie et al. (5), and DNA was purified by two cycles of CsCl-ethidium bromide density gradient ultracentrifugation. Plasmid DNA was digested with *Sal*I restriction endonuclease (Boehringer Mannheim, Indianapolis, Ind.) as specified by the manufacturer, and the fragments were separated by agarose gel electrophoresis (AGE).

Amplification of *virF* and IS*1* sequences. The nucleotide sequence of primers I, II, IV, and V, used to amplify *virF* sequences, and primers VI and VII, used to amplify IS*1* sequences, were obtained from the published DNA sequences of *virF* (39) and IS*1* (35). The orientations of the *virF* primers relative to the *virF* gene are shown in Fig. 6 and consisted of the following: primer I (5′-GCAAATACT TAGCTTGTTGCACAGAG-3′) was 26 bases in length and started at base 1, primer II (5′-CGATGAAGGGCAAAT TGC-3′) was 18 bases in length and started at base 316, primer IV (5′-GTTGAAATGAATAAATTGGTT-3′) was 21 bases in length and started at base 470, and primer V (5′-GGGCTTGATATTCCGATAAGTC-3′) was 22 bases in length and started at base 906. IS*1* oligomers comprised the following: primer VI (5′-GAGGTGCTCCAGTGGCTTCTG 3′, sense primer) was 21 bases in length and started at base 45, and primer VII (5′-GCCCGATGACTTTGTCATG CAGC-3′, antisense primer) was 23 bases in length and started at base 724. An additional oligomer, designated primer III (5′-CAGCGCTATCTCTGCTCTC-3′), was 19 bases in length and started at base 138 of the IS*1*SFO (see Fig. 6), as determined by DNA sequence analysis.

Polymerase chain reactions (PCRs) were done with a DNA Thermal Cycler and GeneAmp DNA amplification kit (The Perkin-Elmer Cetus Corp., Norwalk, Conn.). All reactions amplified the target DNA by using 30 cycles, each cycle consisting of 1 min at 94°C, 1 min at 48°C, and 1 min at 72°C. Fifty picomoles of each primer and 1 to 5 ng of template were used per reaction. Plasmid pRR134, which carries the IS*1* element of R plasmid NR1 (37), was used as the template to amplify IS*1* sequences. The *virF* sequences were amplified from *Shigella* invasion plasmids isolated as described by Birnboim (2).

DNA hybridization. Preparation of filters for in situ colony hybridization was done as previously described (25). Invasion and recombinant plasmid DNA molecules, digested with *Sal*I and separated by AGE, were transferred to nitrocellulose as described by Southern (50). Hybridization analyses of bacterial colony blots and Southern blots of plasmid DNA fragments and PCR-amplified DNA sequences were done under stringent DNA hybridization conditions (55). Insert fragments from recombinant plasmids pHC17 and pEC14 (56) were used as the source of the *ipa* gene probes. DNA sequences were radiolabeled with [α-^{32}P]dCTP by nick translation with a kit from New England Nuclear Corp. (Boston, Mass.).

DNA sequencing. A 1,686-bp sequence, which was PCR amplified by primers I and IV from a 2457O strain isolated in 1990, was inserted into pCR1000 (Invitrogen Corp., San Diego, Calif.) and transformed into *E. coli* INVαF′ cells, generating INVαF′(pWR630). The insert fragment contained on pWR630 was completely sequenced in both directions (41) from a double-stranded DNA template by using the Sequenase DNA sequencing kit from United States Biochemical (Cleveland, Ohio).

Plasmid pWR600 construction. Plasmid pMYSH6500 con-

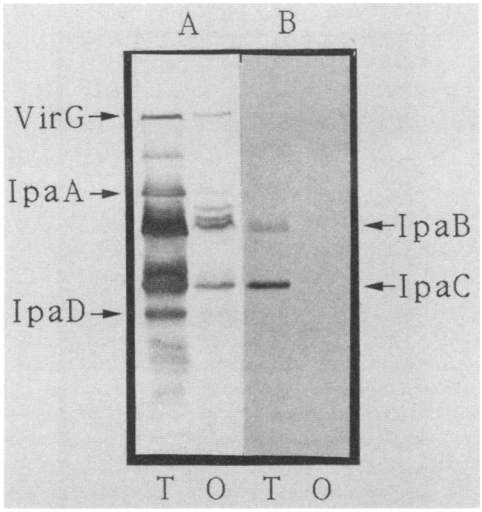

FIG. 1. Immunoblot analysis of Ipa and VirG polypeptides synthesized by *S. flexneri* 2457T and 2457O. Polypeptides from SDS whole-cell lysates of 2457T (T) and 2457O (O) were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. The filters were then screened with convalescent human antiserum that contained antibodies recognizing IpaA, IpaB, IpaC, IpaD, and VirG polypeptides (A) or a mixture of IpaB- and IpaC-specific monoclonal antibodies (B).

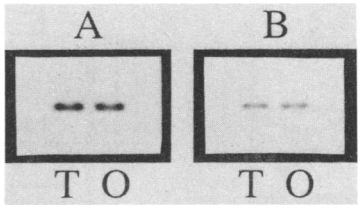

FIG. 2. Hybridization of *ipa*-specific gene sequences to invasion plasmid DNA from *S. flexneri* 2457T and 2457O. Plasmid DNA from 2457T (T) and 2457O (O) was digested with *Eco*RI or *Hin*dIII, separated by AGE, transferred to nitrocellulose, and hybridized to either a 4.7-kb *Hin*dIII *ipaBCD'* probe (A) or an 8-kb *Eco*RI *ipaDAR* probe (B). Only one DNA fragment from each digest hybridized to the probes.

tains the *virF* gene from *S. flexneri* 2a strain YSH6000 on an 8.5-kb fragment (38). A 1,260-bp *Hpa*II fragment from pMYSH6500 was subcloned into *Cla*I-cleaved pBR322 and transformed in *E. coli* HB101, generating HB101(pWR600).

Transduction experiments. 2457T and 2457O strains were screened for their ability to use glycerol or DL-α-glycerophosphate as a sole carbon source for growth by plating them on M9 minimal plates (25) supplemented with nicotinic acid (2 μg/ml) and modified by replacing the glucose with either 20 mM glycerol or 40 mM DL-α-glycerophosphate. The 2457O isolate unable to use glycerol but able to use DL-α-glycerophosphate for growth was transduced with a P1*vir* phage lysate generated from a 2457T strain that was able to use glycerol as its sole carbon source for growth. Transductions were performed as described by Miller (28).

RESULTS

Characterization of 2457O. 2457O colonial variants were unable to bind Congo red dye (36a), express the *ipaBCDA* genes (Fig. 1) (17), synthesize wild-type levels of the VirG polypeptide (Fig. 1), lyse sheep erythrocytes, invade HeLa cells, form plaques in HeLa cell monolayers, or elicit a keratoconjunctivitis reaction in guinea pigs. As previously reported (22), the variation of 2457T to 2457O occurred spontaneously once in 10^4 cell divisions, and in vitro reversion from 2457O to 2457T was not observed. In addition, the colonial morphology of 2457O and 2457T remained stable at growth temperatures between 25 and 42°C.

Expression of the *ipaBCD* genes is required for the invasive phenotype in *Shigella* spp. (45). Southern analysis of 2457O and 2457T invasion plasmid DNA was performed to determine whether the lack of *ipa* gene expression (Ipa^-) in 2457O was due to a deletion or rearrangement of the *ipa* genes. The results of these experiments showed that the *ipa* gene probes recognized similar invasion plasmid DNA fragments in 2457O and 2457T. A 4.7-kb *Hin*dIII fragment of the invasion plasmid from 2457O and 2457T hybridized with a 4.7-kb *Hin*dIII probe that carried the *ipaBCD'* (*D'* refers to a truncated *ipaD* sequence) gene sequence (Fig. 2). Likewise, an 8-kb *Eco*RI probe, which carries a *ipaDAR* gene sequence, hybridized with an 8-kb fragment from a *Eco*RI plasmid digest of 2457O and 2457T. These data indicated that although the *ipa* genes were not expressed in 2457O, they were not mutated by a large genetic alteration. Since the *ipa* genes are known to be coordinately regulated (4, 40), the possibility that the Ipa^- phenotype of 2457O resulted from a regulatory mutation that affected *ipa* gene expression existed.

Plasmid pWR110 restores *ipa* gene expression in 2457O. To determine whether a mutated locus on the invasion plasmid might be responsible for the lack of *ipa* gene expression in 2457O, a functional invasion plasmid was introduced via conjugation into 2457O. Attempts to cure the resident invasion plasmid from 2457O prior to mating, using serial passages, high growth temperatures (51), sodium dodecyl sulfate (SDS) (53), acridine orange (28), and various combinations of these methods, were unsuccessful as monitored by plasmid analysis of the segregants (data not shown). In contrast, 2457T was readily cured of the invasion plasmid by these techniques. Plasmid pWR110, a Tn*5*-tagged invasion plasmid of *S. flexneri* 5 strain M90T that is known to confer the invasive phenotype to plasmid-cured *Shigella* derivatives (43), was transferred from 7262-1-10 (42) into two different 2457O strains, one isolated in 1954 and the other isolated in 1990, and plated on MEA-kanamycin medium. 2457O(pWR110-R64*drd*11) transconjugants that had regained the translucent colonial morphology, the capacity to express the IpaB and IpaC proteins on their surface (Fig. 3), and the ability to invade HeLa cells were isolated. These results indicated that the defect suppressing *ipa* gene expression in 2457O does not result from a chromosomal mutation, since a functional invasion plasmid (pWR110) restored the invasive phenotype in the presence of the 2457O chromosomal background.

The *virF* gene in 2457O contains an IS*1*-like element. The capacity of pWR110 to restore invasion to 2457O suggested that the mutation in 2457O resided on its invasion plasmid. AGE of *Sal*I-digested plasmid DNA from 2457T and two 2457O variants, one isolated in 1954 and the other isolated in 1990, showed that the 13.5-kb *Sal*I fragment in 2457T had increased in size in both 2457O isolates (Fig. 4). The *Sal*I digests from 2457T and 2457O, which contain fragments from a 210-kb invasion plasmid and 160-kb cryptic plasmid (21), and the *Sal*I digest of the 230-kb invasion plasmid pMYSH6000 from *S. flexneri* 2a strain YSH6000 (46), are

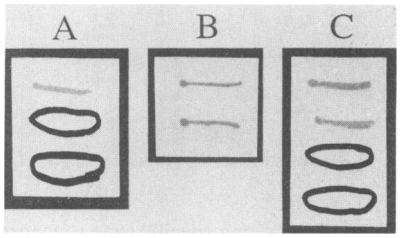

FIG. 3. Immunocolony assay of IpaC polypeptide synthesized by *S. flexneri* 2457T, 2457O, and 2457O derivatives. (A) From top to bottom, 2457T, 2457O-54 (isolated in 1954), and 2457O-90 (isolated in 1990); (B) transconjugants 2457O-54(pWR110) and 2457O-90 (pWR110); (C) transformants 2457O-54(pWR600), 2457O-90(pWR 600), 2457O-54(pBR322), and 2457O-90(pBR322). All strains were streaked on the surface of nitrocellulose overlaid on MEA containing the appropriate antibiotic (none [A], kanamycin [B], or ampicillin [C]) and incubated overnight at 37°C. The filters were then lifted and screened with IpaC-specific monoclonal antibody. Ovals show positions of nonreactive *Shigella* strains.

different. However, the *Sal*I fragment in pMYSH6000 carrying the *virF* gene, an activator of several virulence-related genes (7, 19, 39, 40), is similar in size to the 13.5-kb *Sal*I fragment in 2457T. This similarity and the possibility that an altered *virF* could account for the avirulent phenotype in 2457O suggested that the *virF* gene in 2457O may be contained on the *Sal*I-fragment that increased in size. Southern blot analysis using a PCR-amplified *virF* sequence as a DNA probe confirmed that the *Sal*I fragment carrying the *virF* gene in both 2457O isolates had increased in size relative to its counterpart in 2457T (Fig. 4).

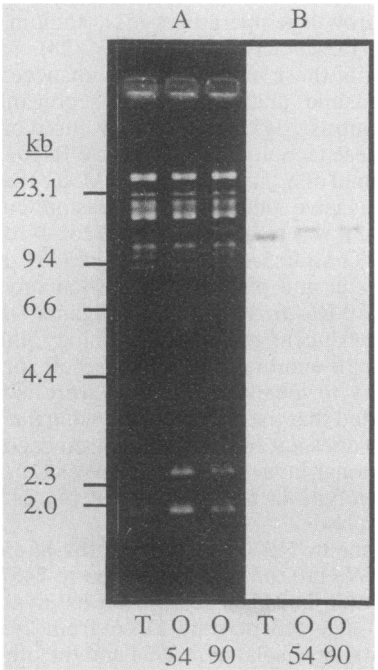

FIG. 4. Analysis of *Sal*I-digested invasion plasmid fragments of *S. flexneri* 2457T and 2457O. Plasmid DNAs from 2457T (T) and two independently isolated 2457O strains, 2457O-54 (O54) and 2457O-90 (O90), were digested with *Sal*I, separated by agarose gel electrophoresis, stained with ethidium bromide (A), transferred to nitrocellulose, and hybridized with a PCR-amplified *virF* DNA probe (B).

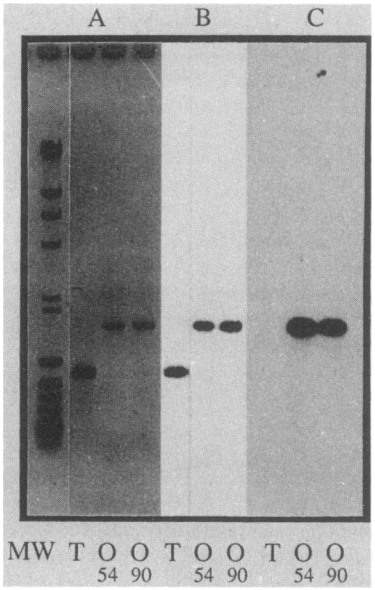

FIG. 5. Comparison of *virF* sequences amplified from *S. flexneri* 2457O and 2457T. Primers I and V were used (see Fig. 6) to PCR amplify *virF* sequences from 2457O-54 (O54), 2457O-90 (O90), and 2457T (T). Amplified sequences were isolated by AGE, stained with ethidium bromide (A) (MW, λ DNA-*Hin*dIII–φX-174 DNA-*Hin*cII digest), transferred to nitrocellulose, and hybridized with either a PCR-amplified 2457T *virF* sequence (B) or a PCR-amplified IS*1* sequence (C).

The size increase of the *virF*-containing *Sal*I fragment implied that the 2457O *virF* gene might have undergone a mutation. To analyze the *virF* gene in more detail, primers I and V were used to amplify *virF*-specific sequences from 2457T and 2457O by PCR. AGE revealed that *virF* sequences amplified from the 2457O isolates were 780 bp larger than those amplified from 2457T (Fig. 5). Since *S. flexneri* contains more than 40 copies of a sequence that hybridizes to the insertion element IS*1* (31), which is 768 bp long (35), the amplified *virF* sequences were probed with an IS*1* sequence amplified from plasmid pRR134 (37). The IS*1* DNA probe hybridized to *virF* sequences amplified from 2457O isolates but not those from 2457T (Fig. 5). However, attempts to amplify the insertion element from PCR-amplified 2457O *virF* sequences with the set of primers used to amplify the pRR134 IS*1* sequence were unsuccessful, suggesting that the insertion element in the 2457O *virF* gene was an IS*1*-like or iso-IS*1* (34) element.

Further PCR analysis of 2457O, using primers II and IV to amplify smaller segments of the *virF* sequence, mapped the iso-IS*1* element within the open reading frame of the *virF* gene. On the basis of the position of *virF* primers II and IV (Fig. 6), all nine 2457O strains contained an iso-IS*1* element inserted between bp 137 and 253 downstream from the 5′ end of the *virF* opening reading frame (Fig. 7).

DNA sequence analysis of the insertion element and insertion site in the 2457O *virF* gene. DNA sequence analysis of the 2457O *virF* locus carried by pWR630 identified a 768-bp insertion element. The insertion element was named IS*1*SFO (*S. flexneri* opaque variants) and was identical to the iso-IS*1* element IS*1*F (34) [recently renamed IS*1*(SF) (54)] isolated from *S. flexneri*, except for five base substitutions. Nucleotides A (position 36), A (position 94), C (position 105), A (position 132), and C (position 445) in IS*1*F (34) were

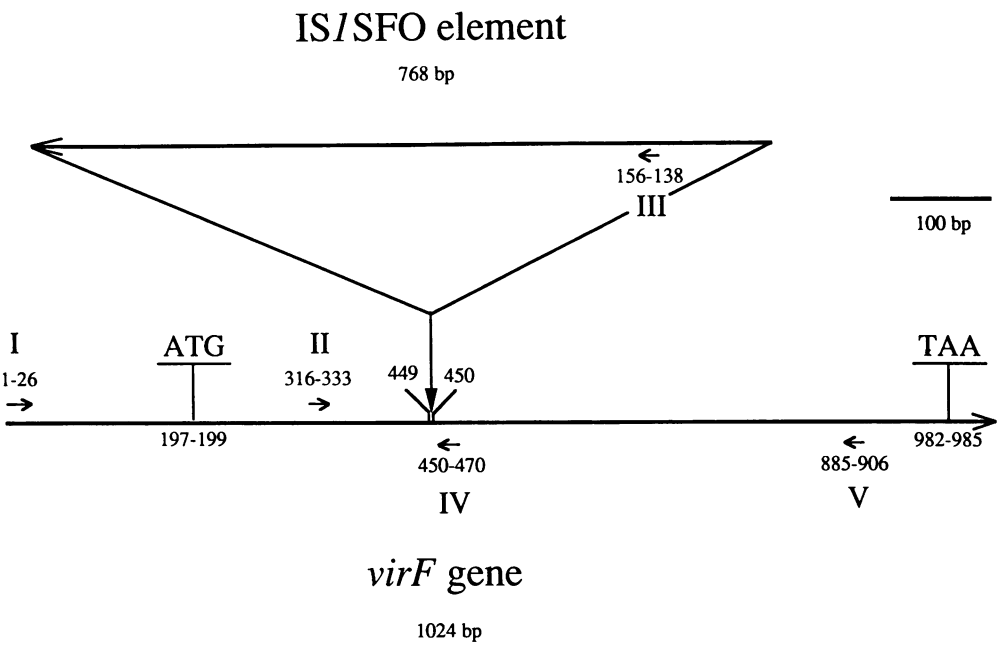

FIG. 6. Map position of IS*1*SFO insertion element within the *virF* gene of *S. flexneri* 2457O. Map positions of the primers (I, II, III, IV, and V), ATG start codon, TAA stop codon, and IS*1*SFO element of the *virF* gene are given. Orientations of primers, insertion element, and gene are indicated by arrows.

replaced in IS*1*SFO by nucleotides G, T, T, G, and A, respectively. Six base pair substitutions in IS*1*SFO relative to IS*1*R (35) were located in regions corresponding to the IS*1* primers VI and VII, which may explain our inability to amplify IS*1*SFO sequences with this set of primers.

DNA sequence analysis of the insertion site in pWR630 identified a 9-bp *virF* target sequence (5′-GAATAATGG-3′) that was duplicated on both sides of IS*1*SFO. IS*1*SFO was integrated into an A · T-rich region immediately after the 3′ G in the target sequence, located 254 bp downstream from the 5′ end of the open reading frame (Fig. 6). IS*1*SFO contained terminal inverted repeats in which 22 of 24 bases from each terminus could form normal hydrogen-bonded pairs. Moreover, the 6 most distal bases of the terminal repeats were homologous to the 6 bases on the 3′ end of the target sequence. The orientation of IS*1*SFO within the *virF* gene is shown in Fig. 6.

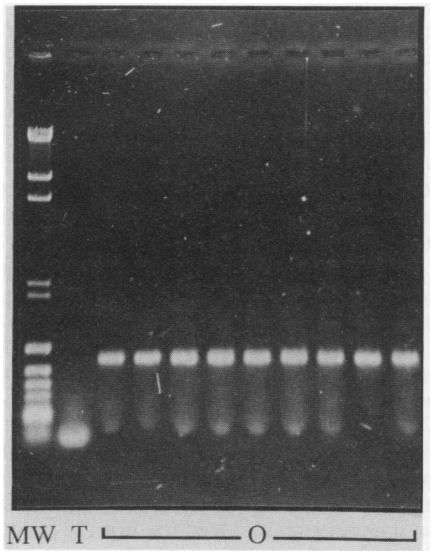

FIG. 7. Analysis of amplified *virF* sequences from nine independently isolated *S. flexneri* 2457O strains. The *virF* sequences were amplified from 2457T (T) and nine 2457Os (O) by primers II and IV, which are complementary to sequences within the opening reading frame of the *virF* gene (Fig. 6). PCR products (MW, λ DNA-*Hin*dIII–φX-174 DNA-*Hin*cII digest) were visualized by AGE and ethidium bromide staining.

The orientation and insertion site of IS*1*SFO is the same in nine 2457O isolates. PCR amplification with *virF* primers I and III established the orientations and map positions of IS*1*SFO in eight other 2457O isolates. The amplification of *virF*-IS*1*SFO DNA by these primers and the orientation of primer III in IS*1*SFO relative to primer I in *virF* (Fig. 6) demonstrated that all of the IS*1*SFO insertions in the 2457O strains were in the same orientation as IS*1*SFO in pWR630. Within the limits of AGE resolution, all nine 2457O isolates amplified an approximately 1,080-bp sequence, which correlated with the size determined from the sequence of pWR630 (data not shown). It was noted that no sequence was amplified from 2457T with this set of primers.

Plasmid pWR600 restores the virulent phenotype to 2457O. To determine whether a functional *virF* gene could restore the virulent phenotype to 2457O, pWR600, carrying a 1,260-bp *Hpa*II fragment encoding a wild-type *virF* gene, was transformed into each 2457O isolate. All 2457O(pWR 600) transformants were able to bind Congo red dye, express *ipaBCDA* (Fig. 3) and *virG* genes, lyse sheep erythrocytes, invade HeLa cells, form plaques in HeLa cell monolayers, and elicit a keratoconjunctivitis reaction in guinea pigs. In addition to restoring the virulent phenotype, pWR600 was able to restore the translucent colonial morphology to all nine independently isolated 2457O hosts.

2457O GlpK$^+$ transductants and revertants are noninvasive. To examine an earlier observation that associated the GlpK$^+$ phenotype with restoration of invasiveness in 2457O, we repeated the previously described transduction and reversion experiments (20). Since strains used in the previous report were unavailable, only the 2457O isolates used in this study were tested. One 2457O variant from 1964 was unable to use glycerol but was able to use DL-α-glycerophosphate as its sole carbon source for growth, which is typical of a *glpK* mutation (20). All other 2457O and 2457T isolates were able to use glycerol or DL-α-glycerophosphate as a sole carbon source for growth. The 1964 isolate of 2457O was transduced with a P1*vir* phage lysate prepared on 2457T, and GlpK$^+$ transductants were selected on plates containing glycerol as the sole carbon source. One hundred transductants were isolated, but none were able to bind Congo red dye, express *ipa* genes, or invade HeLa cells. All of the transductants also maintained their opaque colonial morphology. In addition, no change was detected in the virulence or colonial morphology of 37 spontaneously isolated 1964 2457O GlpK$^+$ revertants.

DISCUSSION

An examination of the noninvasive phenotype of strain 2457O, an opaque colonial variant of *S. flexneri* serotype 2a strain 2457T, has revealed that the avirulent phenotype in 2457O (22) was due to a mutated *virF* gene. The 2457O *virF* mutation resulted from the insertion of an iso-IS*1* element (34), designated IS*1*SFO, within the *virF* open reading frame, thereby preventing the *virF* gene product from activating expression of the *ipa* and other virulence-associated genes. Previous studies have demonstrated that the insertion of an IS*1* element within a gene abolishes expression of that gene (18, 24, 49). The insertional inactivation of *virF* by IS*1*SFO probably occurred at the translational level because of the presence of nonsense codons in all three reading frames of the IS*1*SFO primary sequence and in the primary sequence of other iso-IS*1* elements (34).

The IS*1*SFO element that inserted into *virF* in 2457O may have originated from either the bacterial chromosome or any of the four plasmid species harbored by 2457T. *S. flexneri* has more than 40 copies of an IS*1*-hybridizing element (31), with two to five copies located on the invasion plasmid (3, 8, 46) and one element closely linked to the *virF* gene (7, 38). Why the *virF* gene appears to be a hot spot for IS*1*SFO insertions is unknown; however, the *virF* nucleotide sequence provides a number of IS*1* integration site requirements. For instance, while IS*1* exhibits no apparent preference for a particular target sequence, insertion of this element is not entirely random. IS*1* typically inserts into sequences relatively rich in A · T base pairs, next to G · C base pairs, and near short regions of homology with the ends of the insertion element (15). The location of the IS*1*SFO insertion in the *virF* gene matches these target parameters closely. Although a more recent report questions the role that local homology of the insertion ends plays in determining insertion hot spots (59), the homology between the 6 most terminal bases on the ends of the IS*1*SFO element and the 6 bases on the 3′ end of the target sequence is striking. There was a strong orientation effect on IS*1*SFO integration such that all of the IS*1*SFO insertions mapped in one orientation; a similar phenomenon has been reported with other IS*1* insertion mutations (59).

Transfer of a functional *virF* gene was able to restore *ipa* gene expression and invasiveness to 2457O. Synthesis of the VirG polypeptide was restored to wild-type levels and the translucent colonial morphology of the parental strain was reestablished in the 2457O *virF*$^+$ transformants. Although the VirF polypeptide activates transcription of the *ipa* and *virG* genes (40), the degree of control exerted on these genes by VirF appears to be different. The transition from 2457T to 2457O, with concomitant loss of Ipa polypeptide synthesis and reduced synthesis of the VirG polypeptide, indicates that VirF tightly regulates *ipa* gene expression, whereas VirG synthesis is only partly dependent on VirF. The residual expression of *virG* in 2457O may be the result of the chromosomal locus *kcpA*, which like *virG* is required for intercellular bacterial spreading and keratoconjunctivitis provocation (1, 12, 23, 36, 58). The *kcpA* locus has been proposed to be a regulator of *virG* expression (36) and presumably is functional in 2457O.

The relationship of *virF* expression to colony morphology is not clear. Surface-exposed Ipa polypeptides are not synthesized at 30°C (27); however, the colonial morphology of 2457T and 2457O was unaffected by growth temperatures between 25 and 42°C; therefore, it is unlikely that Ipa polypeptides play a role in determining either the opaque or translucent colony type. At 30°C the *virF* gene is transcribed at a reduced level relative to its transcription at 37°C (52), and it is conceivable that this low-level expression is sufficient to activate transcription of an uncharacterized gene(s) that expresses a surface component(s) responsible for the translucent colonial morphology. Alternatively, VirF may be the sole determinant of the translucent phenotype.

The ability of a wild-type *virF* gene to restore virulence to 2457O appears contradictory to the results of a previous report that described an association between the GlpK$^+$ phenotype and the restoration of invasion in 2457O (20). However, there is the possibility that a GlpK$^+$-linked locus is involved in the precise excision of the IS*1*SFO element from the *virF* gene of 2457O, which could then restore expression of the *ipa* genes and the invasive phenotype. On the basis of the noninvasive phenotype of the 2457O GlpK$^+$ transductants and revertants generated in this study, it does not appear that the GlpK$^+$ phenotype directly contributes to the restoration of the virulent phenotype in 2457O. Although unlikely, there remains the possibility that the 2457O isolate used in the previous study (20) contained a mutation different from what has been found in the nine 2457O isolates analyzed in this report.

Without the capacity to produce symptoms or pathology, 2457O was considered as one of the first attenuated vaccine candidates (22). When given in multiple doses to rhesus monkeys, 2457O was found to be a safe and effective oral vaccine against a challenge by 2457T (14). However, in a human safety trial, 2457O caused shigellosis in more than a third of those who ingested 10^{10} cells in the initial dose (9). Characterization of *Shigella* isolates obtained from the stool cultures of three of these infected individuals revealed that these 2457O virulent revertants had regained the translucent colonial morphology and lost the insertion element normally present in the 2457O *virF* gene (29). These observations suggest that IS*1*SFO was excised from the 2457O *virF* gene in a precise manner that restored the normal function of *virF* to the cells.

The insertion of IS*1*SFO into *virF* had widespread effects on several phenotypes and may be associated with the remarkable stability of the 2457O invasion plasmid. The IS*1*SFO insertion into *virF* could stabilize the invasion plasmid when 2457O is outside of the host, whereas the excision of IS*1*SFO from *virF* allows 2457O to revert to a

virulent strain that can invade the colonic epithelium when inside the host. This suggests that the relationship between IS*1*SFO and the *virF* gene could be beneficial to the organism. *Shigella* strains propagated outside of the host often cure the invasion plasmid or delete virulence-essential regions of their invasion plasmids (6, 26, 46), thereby conserving energy that would normally be expended on maintaining virulence genes not essential for survival outside of the host. These avirulent strains, however, are unlikely to revert to a virulent phenotype and so cannot exploit the host environment if subsequently ingested. Thus, unlike its counterparts harboring genetically cured or altered invasion plasmids, the capacity of 2457O to maintain its essentially unaltered invasion plasmid may provide 2457O with a selective advantage.

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