Spontaneous Insertion of an ISL-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 10⁸ cell divisions, with concomitant loss of ipa gene expression and virulence. The appearance of 24570 was associated with the insertional inactivation of virF, an invasion plasmid-encoded positive regulator of ipa gene expression. Plasmid pWR110, a Tn5-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 Safl 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 ISI DNA probe whereas the amplified 2457T virF sequence did not. DNA sequence analysis mapped the insertion element, designated IS1/SFO, within an AT-rich region of the virF open reading frame and identified a 9-bp virF target sequence that was duplicated at the insertion site of IS1/SFO. The DNA sequence of IS1/SFO was >99% homologous to IS1/F. Plasmid pWR600, carrying a 1,260-bp HpalI 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 dysenteric 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 BamH1 and EcoRI 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 glyceral 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 ISL-like element inserted into the open reading frame of the invasion plasmid-encoded virF gene.
Insertional inactivation of the virF gene in 24570 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 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 10⁴ cell divisions (22). Shigella strains were routinely tested in an agglutination reaction with S. flexneri 2a-specific antisera 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 Tn5-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 (R64drd11) 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 SalI 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 IS1 sequences. The nucleotide sequence of primers I, II, IV, and V, used to amplify virF sequences, and primers VI and VII, used to amplify IS1 sequences, were obtained from the published plasmid DNA sequences of virF (39) and IS1 (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'-GCAATATCTAGCTTGTTCACAGG-3') was 26 bases in length and started at base 1, primer II (5'-CGATGAAGGGCCAATTGTC-3') was 18 bases in length and started at base 316, primer IV (5'-GTTGAAATGATAAATTGGTT-3') was 21 bases in length and started at base 470, and primer V (5'-CTTATCCGCATATAAGTCG-3') was 22 bases in length and started at base 906. IS1 oligomers comprised the following: primer VI (5'-GAGGGTCGTCCAGTGGCCTCTG3', sense primer) was 21 bases in length and started at base 45, and primer VII (5'-GCGCGATCTTTGTGCAATCACG-3', antisense primer) was 23 bases in length and started at base 724. An additional oligomer, designated primer III (5'-CACCGCTATCTCCTGCTTC-3'), was 19 bases in length and started at base 138 of the IS1/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 IS1 element of R plasmid NR1 (37), was used as the template to amplify IS1 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 SalI and separated by AGE, were transferred to nitrocellulose as described by Southern (30). 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 pCH17 and pEC14 (56) were used as the source of the ipa gene probes. DNA sequences were radiolabeled with [α-32P]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 INVaF' cells, generating INVaF'(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-
tains the virF gene from S. flexneri 2a strain YSH6000 on an 8.5-kb fragment (38). A 1,260-bp HpaII fragment from pMYSH6500 was subcloned into ClaI-cleaved pBR322 and transformed in E. coli HB101, generating HB101(pWR600).

Transduction experiments. 2457T and 24570 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 24570 isolate unable to use glycerol but able to use DL-α-glycerophosphate for growth was transduced with a P1vir 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 24570. 24570 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 24570 occurred spontaneously once in 10⁶ cell divisions, and in vitro reversion from 24570 to 2457T was not observed. In addition, the colonial morphology of 24570 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 24570 and 2457T invasion plasmid DNA was performed to determine whether the lack of ipa gene expression (Ipa−) in 24570 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 24570 and 2457T. A 4.7-kb HindIII fragment of the invasion plasmid from 24570 and 2457T hybridized with a 4.7-kb HindIII probe that carried the ipaBCD' probe (A) or an 8-kb EcoRI ipaDAR probe (B). Only one DNA fragment from each digest hybridized to the probes.

Plasmid pWR110 restores ipa gene expression in 24570. To determine whether a mutated locus on the invasion plasmid might be responsible for the lack of ipa gene expression in 24570, a functional invasion plasmid was introduced via conjugation into 24570. Attempts to cure the resident invasion plasmid from 24570 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 and invasion assays (data not shown). In contrast, 2457T was readily cured of the invasion plasmid by these techniques. Plasmid pWR110, a Tn5-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 24570 strains, one isolated in 1954 and the other isolated in 1990, and plated on MEA-kanamycin medium. 24570(pWR110-R64drd11) 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 24570 does not result from a chromosomal mutation, since a functional invasion plasmid (pWR110) restored the invasive phenotype in the presence of the 24570 chromosomal background.

The virF gene in 24570 contains an ISL-like element. The capacity of pWR110 to restore invasion to 24570 suggested that the mutation in 24570 resided on its invasion plasmid. AGE of SalI-digested plasmid DNA from 2457T and two 24570 variants, one isolated in 1954 and the other isolated in 1990, showed that the 13.5-kb SalI fragment in 2457T had increased in size in both 24570 isolates (Fig. 4). The SalI digests from 2457T and 24570, which contain fragments from a 210-kb invasion plasmid and 160-kb cryptic plasmid (21), and the SalI digest of the 230-kb invasion plasmid pMYSH6000 from S. flexneri 2a strain YSH6000 (46), are
different. However, the SalI 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 SalI fragment in 2457T. This similarity and the possibility that an altered virF could account for the avirulent phenotype in 24570 suggested that the virF gene in 24570 may be contained on the SalI-fragment that increased in size. Southern blot analysis using a PCR-amplified virF sequence as a DNA probe confirmed that the SalI fragment carrying the virF gene in both 24570 isolates had increased in size relative to its counterpart in 2457T (Fig. 4).

FIG. 3. Immunoclonal assay of IpaC polypeptide synthesized by S. flexneri 2457T, 24570, and 24570 derivatives. (A) From top to bottom, 2457T, 24570-54 (isolated in 1954), and 24570-90 (isolated in 1990); (B) transconjugants 24570-54(pWR110) and 24570-90 (pWR110); (C) transfectants 24570-54(pWR600), 24570-90(pWR600), 24570-54(pBR322), and 24570-90(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.

The size increase of the virF-containing SalI fragment implied that the 24570 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 24570 by PCR. AGE revealed that virF sequences amplified from the 24570 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 IS1 (31), which is 768 bp long (35), the amplified virF sequences were probed with an IS1 sequence amplified from plasmid pRR134 (37). The IS1 DNA probe hybridized to virF sequences amplified from 24570 isolates but not those from 2457T (Fig. 5). However, attempts to amplify the insertion element from PCR-amplified 24570 virF sequences with the set of primers used to amplify the pRR134 IS1 sequence were unsuccessful, suggesting that the insertion element in the 24570 virF gene was an IS1-like or iso-IS1 (34) element.

Further PCR analysis of 24570, using primers II and IV to amplify smaller segments of the virF sequence, mapped the iso-IS1 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 24570 strains contained an iso-IS1 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 24570 virF gene. DNA sequence analysis of the 24570 virF locus carried by pWR630 identified a 768-bp insertion element. The insertion element was named IS1/SFO (S. flexneri opaque variants) and was identical to the iso-IS1 element ISF (34) [recently renamed IS1(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 ISF (34) were...
replaced in IS/SFO by nucleotides G, T, T, G, and A, respectively. Six base pair substitutions in IS/SFO relative to IS/R (35) were located in regions corresponding to the ISI primer termini VI and VII, which may explain our inability to amplify IS/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/SFO. IS/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/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/SFO within the virF gene is shown in Fig. 6.

The orientation and insertion site of IS/SFO is the same in nine 24570 isolates. PCR amplification with virF primers I and III established the orientations and map positions of IS/SFO in eight other 24570 isolates. The amplification of virF-IS/SFO DNA by these primers and the orientation of primer III in IS/SFO relative to primer I in virF (Fig. 6) demonstrated that all of the IS/SFO insertions in the 24570 strains were in the same orientation as IS/SFO in pWR630. Within the limits of AGE resolution, all nine 24570 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 24570. To determine whether a functional virF gene could restore the virulent phenotype to 24570, pWR600, carrying a 1,260-bp HpaII fragment encoding a wild-type virF gene, was transformed into each 24570 isolate. All 24570(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 24570 hosts.
24570 GlpK+ transductants and revertants are noninvasive. To examine an earlier observation that associated the GlpK+ phenotype with restoration of invasiveness in 24570, we repeated the previously described transduction and reversion experiments (20). Since strains used in the previous report were unavailable, only the 24570 isolates used in this study were tested. One 24570 variant from 1964 was unable to use glycerol but was able to use D,L-a-glycerophosphate as its sole carbon source for growth, which is typical of a glpK mutation (20). All other 24570 and 2457T isolates were able to use glycerol or D,L-a-glycerophosphate as a sole carbon source for growth. The 1964 isolate of 24570 was transduced with a Plvir 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 24570 GlpK+ revertants.

DISCUSSION

An examination of the noninvasive phenotype of strain 24570, an opaque colonial variant of S. flexneri serotype 2a strain 2457T, has revealed that the avirulent phenotype in 24570 (22) was due to a mutated virF gene. The 24570 virF mutation resulted from the insertion of an iso-ISJ element (34), designated ISJ/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 ISJ element within a gene abolishes expression of that gene (18, 24, 49). The insertional inactivation of virF by ISJ/SFO probably occurred at the translational level because of the presence of nonsense codons in all three reading frames of the ISJ/SFO primary sequence and in the primary sequence of other iso-ISJ elements (34).

The ISJ/SFO element that inserted into virF in 24570 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 ISJ-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 ISJ/SFO insertions is unknown; however, the virF nucleotide sequence provides a number of ISJ integration site requirements. For instance, while ISJ exhibits no apparent preference for a particular target sequence, insertion of this element is not entirely random. ISJ 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 ISJ/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 ISJ/SFO element and the 6 bases on the 3' end of the target sequence is striking. There was a strong orientation effect on ISJ/SFO integration such that all of the ISJ/SFO insertions mapped in one orientation; a similar phenomenon has been reported with other ISJ insertion mutations (59).

Transfer of a functional virF gene was able to restore ipa gene expression and invasiveness to 24570. Synthesis of the VirG polypeptide was restored to wild-type levels and the translocute colonial morphology of the parental strain was reestablished in the 24570 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 24570, 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 24570 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 24570.

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 24570 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 translocute colonial morphology. Alternatively, VirF may be the sole determinant of the translocute phenotype.

The ability of a wild-type virF gene to restore virulence to 24570 appears contradictory to the results of a previous report that described an association between the GlpK+ phenotype and the restoration of invasion in 24570 (20). However, there is the possibility that a GlpK+-linked locus is involved in the precise excision of the ISJ/SFO element from the virF gene of 24570, which could then restore expression of the ipa genes and the invasive phenotype. On the basis of the noninvasive phenotype of the 24570 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 24570. Although unlikely, there remains the possibility that the 24570 isolate used in the previous study (20) contained a mutation different from what has been found in the nine 24570 isolates analyzed in this report.

Without the capacity to produce symptoms or pathology, 24570 was considered as one of the first attenuated vaccine candidates (22). When given in multiple doses to rhesus monkeys, 24570 was found to be a safe and effective oral vaccine against a challenge by 2457T (14). However, in a human safety trial, 24570 caused shigellosis in more than a third of those who ingested 1010 cells in the initial dose (9). Characterization of Shigella isolates obtained from the stool cultures of three of these infected individuals revealed that these 24570 virulent revertants had regained the translocute colonial morphology and lost the insertion element normally present in the 24570 virF gene (29). These observations suggest that ISJ/SFO was excised from the 24570 virF gene in a precise manner that restored the normal function of virF to the cells.

The insertion of ISJ/SFO into virF had widespread effects on several phenotypes and may be associated with the remarkable stability of the 24570 invasion plasmid. The ISJ/SFO insertion into virF could stabilize the invasion plasmid when 24570 is outside of the host, whereas the excision of ISJ/SFO from virF allows 24570 to revert to a
virulent strain that can invade the colonic epithelium when inside the host. This suggests that the relationship between IS/FPO 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 their counterparts harboring genetically cured or altered invasion plasmids, the capacity of 24570 to maintain its essentially unaltered invasion plasmid may provide 24570 with a selective advantage.

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