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Genetic and Physical Evidence for Plasmid Control of *Shigella sonnei* Form I Cell Surface Antigen

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Virulent *Shigella sonnei* synthesize a surface antigen (form I) which appears to be one of several requirements needed for this host to invade epithelial cells. Upon restreaking on agar media, form I cells readily and irreversibly generate form II cells that lack the form I antigen. All form II cells are avirulent. Plasmid deoxyribonucleic acid of form I and II cells of four different *S. sonnei* isolates, obtained from different areas of the world, was analyzed by agarose gel electrophoresis. A large plasmid (~120 megadaltons in three of the strains) that is present in form I cells was always absent from form II derivatives. Attempts to transfer conjugally only this large plasmid from form I to genetically marked form II cells were unsuccessful. However, a composite molecule, apparently formed by recombination between the large form I plasmid and a self-transmissible plasmid, was found to transfer the form I trait. Transconjugant *S. sonnei* strains acquiring the form I antigen could retransfer this trait to *S. sonnei*, *Shigella flexneri*, or *Salmonella typhi*. These preliminary findings demonstrate that *S. sonnei* form I antigen synthesis is mediated by a large plasmid which is lost spontaneously at a relatively high frequency from *S. sonnei* strains.

Bacteria of the genus *Shigella* remain a major cause of serious gastrointestinal disease in the world. Although the number of *Shigella* infections has been reduced over the past 50 years, over 15,000 cases still occur annually in the United States. Most of these cases occur in mental institutions, on Indian reservations, and among the poor in inner cities. *Shigella sonnei*, one of the four species of this genus, is currently responsible for more than two-thirds of the shigellosis cases reported in the United States. Unlike the species *S. dysenteriae*, *S. flexneri*, and *S. boydii*, each of which is made up of multiple serotypes, *S. sonnei* comprises a single serological type. Freshly isolated, virulent *S. sonnei* strains display a smooth colonial appearance, termed form I, on agar medium. These colonies are unstable and dissociate to rough-appearing colonies termed form II (2). This form variation is associated with the loss of 2-amino-deoxy-L-altruonic acid from the lipopolysaccharide matrix of form I bacilli (6). Form II colonies are uniformly avirulent, and reversion of form II colonies to form I has not been observed. Although the presence of form I antigen is a requisite of virulence, it cannot be the only requirement, because laboratory isolates of form I strains exist which are unable to penetrate epithelial cells.

The form variation that occurs in *S. sonnei* bears some resemblance to the colonial variation (translucent to opaque colonies; T to O) which has been described in other *Shigella* species (3, 4) in that these colonial morphological alterations are essentially irreversible and all result in loss of virulence due to the inability to penetrate epithelial cells. The avirulent colonial variants of *S. dysenteriae* and *S. flexneri*, however, have the same antigenic structure as their virulent parents, whereas the virulent *S. sonnei* express the form I antigen which is lost in form II variants. Furthermore, transitions in colonial morphology of *S. sonnei* are observed much more frequently than the classical T to O colonial variation observed with other shigellae.

Recently, the plasmid content of virulent and avirulent colonial variants of *S. flexneri* 2a was examined to determine whether plasmids were somehow involved in the T to O shift, but no alteration in plasmid content could be detected (7). The purpose of the current study was to examine the plasmids of form I cells and form II variants of *S. sonnei* strains initially isolated in Japan, England, and the United States to see whether form I antigen synthesis is plasmid controlled. The results presented herein indicate that a large plasmid is responsible for the expression of the form I antigen.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table 1. The four parental strains of *S.*

TABLE 1. *Bacterial strains*

Strain designation	Relevant genotype/phenotype[a]	Source
Shigella sonnei		
53G	*lac nad*	Isolated in Japan
50E	*lac nad*	Isolated in Japan
9774	*lac nad*	Isolated in the U. K.
MBI	*lac nad*	Isolated in New York state
772-7 II	*lac nad met* Nal^r	Form II, *met*, Nal^r mutant of 53G
775-14 II	*lac nad his*	Form II, *his* mutant of 53G
Shigella flexneri		
M4243	*lac nad*	Virulent strain isolated in Japan
2457 G1	*lac nad*	Avirulent mutant of M4243
Salmonella typhi		
643 Holland C$^-$	*lac cys trp met his* Str^r	Plasmid-free strain obtained from L. S. Baron
643 F-*lac*	*lac cys trp*/F*lac*	Obtained from L. S. Baron
Escherichia coli K-12		
HU679	*lac thr leu thi* Nal^r/F′(Ts)*lac*::Tn*3*	Obtained from S. Falkow

[a] *S. sonnei* strains require nicotinic acid (*nad*) and are slow lactose fermenters. However, under the conditions of our experiments, none of the wild-type strains formed colonies on minimal medium with lactose as the sole carbon source.

sonnei were form I and displayed typical *S. sonnei* biochemical traits. *Salmonella typhi* 643, harboring a wild-type F*lac* plasmid, served as a plasmid donor in these studies. Similarly, the *Escherichia coli* K-12 strain HU679, which contains an F′ plasmid that is temperature sensitive for replication and carries the genes for lactose utilization as well as a transposon encoding resistance to penicillins (Tn*3*), was also employed. Auxotrophic requirements for either histidine or methionine were introduced into *S. sonnei* 53G by nitrosoguanidine mutagenesis. Chromosomal mutation to nalidixic acid resistance (Nal^r) was selected by plating 10^{10} cells on nutrient agar containing 50 μg of nalidixic acid per ml. These and other genetic characteristics were used to distinguish donor from recipient strains and as selective traits in mating experiments. Strains were preserved in the lyophilized state and maintained on nutrient agar slants during the course of these experiments.

Media and conjugational transfer studies. Cells were routinely grown at 37°C in Pennassay broth (Difco) or on nutrient or minimal agar (1). When necessary, nalidixic acid (50 μg/ml) or ampicillin (Ap; 20 μg/ml), or both, were added to the media. For conjugal deoxyribonucleic acid (DNA) transfer studies, equal amounts (0.1 ml) of overnight broth cultures of the donor and recipient strains were added to 10 ml of Penassay broth. After 24 h of incubation, 3 ml of the mixture was centrifuged, washed in 0.9% saline, suspended in 1 ml of saline, and plated with appropriate dilutions on the proper minimal medium. Successful mating experiments were repeated using a 7-h mixed-cell incubation period. Unsuccessful transfer studies carried out in broth were repeated by mating concentrated donor and recipient cells on a solid medium. Transconjugant clones were always purified three times on minimal medium by serial single-colony isolation. Recipient cell types were verified by selective biochemical tests and with specific antisera. The stability of lactose utilization was monitored, when necessary, on MacConkey agar (Difco), and pertinent biochemical traits were examined by standard diagnostic tests. Some conjugal transfer experiments involving the HU679 donor strain were conducted at 32°C to ensure that the temperature-sensitive F′(Ts)*lac*::Tn*3* plasmid was stably maintained. To select for transposition of Tn*3*, strains that had received the F′(Ts)*lac*::Tn*3* plasmid were incubated at the nonpermissive temperature, 42°C, and selected on MacConkey agar supplemented with ampicillin. The Lac$^-$, ampicillin-resistant (Ap^r) colonies were presumed to have transposed Tn*3* to another molecule and to have lost the F′(Ts)*lac*::Tn*3* plasmid.

Serological tests. Typing serum specific for *S. sonnei* form I antigen was prepared by adsorbing antiserum made against form I cells with form II cells. Form II-specific antiserum was obtained from rabbits immunized with form II cells. *S. flexneri* type 2 and *Salmonella typhi* 9,12 specific sera were obtained from the Biologics Research Department of this Institute. Purified clones were usually scored for antigenic type by slide agglutination tests. Cross-reactions among the parental strains of different species were not observed. Tube agglutination assays were performed as needed. For these tests, equal amounts (0.5 ml) of saline suspensions of agar-grown bacteria were added to twofold serial dilutions of antiserum. Tests were read after 18 h of incubation at 52°C.

Virulence assay. The Sereny test (12), which measures an organism's ability to invade epithelial cells as indicated by the production of keratoconjunctivitis, was used as an indicator of virulence. One drop (~5 × 10^8 cells) of a suspension of bacteria was deposited into the conjunctival sac of guinea pigs, and the eyes were observed over a period of 5 days for a positive reaction. Appropriate positive and negative control tests were always conducted.

DNA isolation and analyses. For qualitative

DNA analysis by agarose gel electrophoresis, two DNA isolation procedures were employed. In one method, cells were grown overnight in 50 ml of Penassay broth to a density of ~5 × 10^9 cells per ml. Plasmid DNA, released from lysozyme-induced spheroplasts via sodium dodecyl sulfate treatment, was purified by salt precipitation and phenol extraction and then precipitated in ethanol (11).

Alternatively, an abbreviated form of the method of Hansen and Olsen (5) was employed. In this procedure, 1 to 3 ml of mid-log-phase cells was collected by centrifugation, lysed with sodium dodecyl sulfate at pH 12.4, and subsequently neutralized. The bacterial chromosomal DNA was removed by salt precipitation, and the plasmid DNA was precipitated with polyethylene glycol. Precipitated plasmid DNA from both procedures was dissolved in 10 mM tris(hydroxymethyl)aminomethane–1 mM ethylenediaminetetraacetic acid (pH 8) and stored at −20°C. DNA obtained by these methods contained a small amount of fragmented chromosomal DNA which, when visible, electrophoresed at a characteristic position in agarose gels.

DNA samples (50 to 100 μl) were electrophoresed through 0.7% agarose vertical slab gels (3 mm thick by 15 cm wide by 12 cm deep) in TBE buffer [89 mM tris(hydroxymethyl)aminomethane, 89 mM boric acid, and 2.5 mM disodium ethylenediaminetetraacetic acid] at 100 V (187 mA) for 3 h. Gels were stained for >2 h in 5 μg of aqueous ethidium bromide per ml, destained for 1 h in water, and then photographed with a Polaroid MP3 camera using type 55P/N film and a no. 9 Wratten filter. Appropriate DNA molecular weight standards were electrophoresed in wells adjacent to uncharacterized DNA preparations, as described previously (7, 10).

RESULTS

Transitions from form I to form II. *S. sonnei* comprises a single serological group normally expressing a form or phase I somatic antigen which contains 2-amino-deoxy-L-altruonic acid. When examined with a low-power stereomicroscope utilizing oblique illumination, form I colonies appear smooth and even-edged. Upon restreaking on most agar media, form I colonies spontaneously give rise, at frequencies as high as 50%, to uneven-edged, rough-appearing form II colonies, which lack the form I antigen. In this study, four representative *S. sonnei* strains isolated from widely separated regions of the world were found to undergo this previously reported colonial morphology alteration (2). This rapid transition is irreversible; *S. sonnei* form II cells have not been observed to revert spontaneously to form I cells. Slide agglutination tests with antisera specific for the form I and form II antigens (see Materials and Methods) were used to verify the transitions that were initially observed with low-power microscopy.

Plasmid analyses of form I and II strains. The rapid, irreversible loss of form I antigen during the transition to form II cell type strongly suggested that a plasmid(s) was involved. Thus, plasmid DNA obtained from each of the form I *S. sonnei* strains was compared to plasmid DNA obtained from a variety of form II derivatives of these strains. Comparison of plasmid content was made by examination of plasmid profiles obtained after agarose gel electrophoresis. The form I strains all contained a large plasmid (Fig. 1, marked by an asterisk) that was lost during the transition to form II colonies. Although the DNA profiles of only four representative isogenic sets of form I and form II colonies are shown, more than 15 colonies of each antigenic type were examined, and virtually identical results were obtained whether the plasmid DNA was isolated by the techniques of Meyers et al. (11) or Hansen and Olsen (5). Furthermore, other form II derivatives of these and other parental *S. sonnei* strains not listed in Table 1 always showed the loss of a large plasmid relative to the form I parent (unpublished data). The fact that the plasmid species marked by asterisks in Fig. 1 are found in fresh DNA preparations which, with age, generate faster-mov-

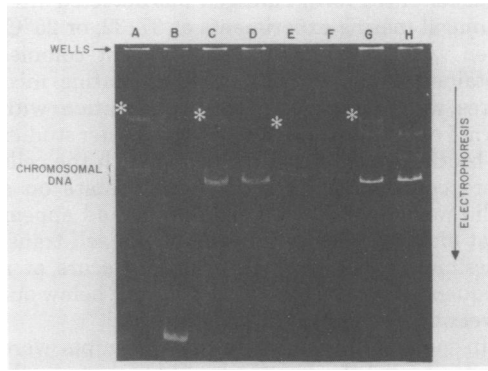

FIG. 1. *Agarose gel electrophoretic profiles of circular plasmid DNA obtained by the Hansen-Olsen technique (5) from sets of isogenic form I and II S. sonnei strains. DNA isolation and gel electrophoresis procedures are given in the text. Plasmid profile of: (A) strain 53G form I; (B) 53G form II; (C) strain 50E form I; (D) 50E form II; (E) strain 9774 form I; (F) 9774 form II; (G) strain MBI form I; and (H) MBI form II. The asterisks mark the large plasmid that is seen in each form I strain but is always absent in the form II derivatives. The DNA in well B was overloaded to verify loss of the large plasmid. Strain 53G carries a plasmid of ~2 Mdal in molecular size, which although not easily seen in DNA from form I strains (well A), was very apparent in the DNA of form II strains (well B). The gel position expected for fragmented chromosomal DNA is indicated. However, small plasmids or linear forms of large plasmids might also band in this area under these electrophoretic conditions.*

ing, apparently linear forms under these electrophoretic conditions substantiates that these are covalently closed, circular molecular species. The plasmid responsible for form I antigen synthesis in strains 53G, 50E, and MBI was estimated by electrophoretic mobility to be 120 megadaltons (Mdal) in size, whereas the analogous plasmid in strain 9774 was slightly smaller (~90 Mdal). In all cases studied, only the large plasmid was lost during the shift from form I to II cells, although one or more smaller plasmids ranging from approximately 2 to 80 Mdal were apparently present in both form I and II cell types of these strains.

Conjugal transfer studies. The large size of the plasmid present in form I cells would imply that it is conjugative, since most large plasmids are self-transmissible. However, we have not been able to correlate any identifiable trait other than form I antigen synthesis with the presence of this large plasmid, making it difficult to monitor plasmid transfer. Initially, attempts were made to detect visually the reversion from form II back to form I colonies after conjugal transfer of the form I plasmid from Met$^-$ donor *S. sonnei* 53G cells to His$^-$ form II 53G recipient cells. No transfer of the form I antigen was detected after conjugal mating experiments at 37, 32, or 25°C, even when several hundred recipient colonies obtained from different conjugal mating mixtures were tested by slide agglutination with form I antisera. Neither conjugal transfer studies in broth for 24 h nor matings in which the cells were concentrated and allowed to mate on a solid medium were successful. It would appear that either the form I plasmid is not self-transmissible or that conjugal transfer occurs at a frequency (10^{-3} to 10^{-2} per donor cell) below our level of detection in this experiment.

In view of the above results, attempts were made to label the form I plasmid with an easily detectable marker, ampicillin resistance, which would allow one to monitor low-frequency (i.e., 10^{-8} to 10^{-9} transconjugants per donor cell) plasmid transfer. To accomplish this, the F′(Ts)-*lac*::Tn*3* plasmid from strain HU679 was first introduced at 32°C into *S. sonnei* form I cells. After purification at 32°C on MacConkey agar containing ampicillin, cells were grown in broth at 42°C for approximately 10 generations before plating on MacConkey agar containing ampicillin. Lac$^-$ Ap^r colonies were assumed to have the Tn*3* unit transposed to either a plasmid or the host chromosome, and the F′(Ts)*lac*::Tn*3* plasmid was presumed to have been lost. Concomitant transfer of Ap^r and the form I antigen trait could now be assessed. When more than 50 different Ap^r Lac$^-$ *S. sonnei* form I strains, constructed as described above, were examined, none was able to donate form I synthesizing ability or Ap^r to appropriate form II recipient cells. These data again suggest that the large form I plasmid is not self-transmissible. Further efforts to label this plasmid with other transposons have ended with similar results; i.e., the form I plasmid does not appear to be conjugative.

Assuming that the large plasmid responsible for form I antigen synthesis is not self-transmissible, one might be able to mobilize this plasmid with other conjugative plasmids. Twenty form I *S. sonnei* 53G isolates were each mated with bacteria containing either an F*lac* plasmid or the RP4 plasmid. *S. sonnei* derivatives carrying either RP4 or the F*lac* plasmid were selected and purified on appropriate media. When these strains were employed as donors in matings with nutritionally distinguishable, form II recipients, F*lac* or RP4 transfer appeared normal, but none of the transconjugants was observed visually or serologically to acquire the form I antigen. Subsequently, experiments were designed to mobilize the form I plasmid by recombination with the F′(Ts)*lac*::Tn*3* plasmid. It was reasoned that the homology between the Tn*3* unit presumed to have been transposed to the form I plasmid in some clones and the Tn*3* unit located on the F′(Ts)*lac*::Tn*3* plasmid would result in recombination between these two plasmids and transfer of the composite molecule. Therefore, 20 Ap^r Lac$^-$ form I *S. sonnei* 53G independent isolates [obtained as described above by eliminating the F′(Ts)*lac*::Tn*3* plasmid at 42°C while selecting for Tn*3* transposition in the presence of ampicillin] were remated with strain HU679. Lac$^+$ Ap^r form I *S. sonnei* derivatives, selected on lactose minimal medium, were purified on MacConkey agar containing ampicillin. These derivatives are presumed to have acquired the F′(Ts)*lac*::Tn*3* plasmid. When 20 different Ap^r Lac$^+$ His$^-$ form I *S. sonnei* 53G strains carrying the F′(Ts)*lac*::Tn*3* plasmid were mated with recipient *S. sonnei* 772-7 II cells, one donor strain (5006-7-3) was observed to transfer form I antigen-synthesizing ability. Although many *S. sonnei* 772-7 recipients grew on the selective lactose minimal medium containing nalidixic acid, indicating that the F′(Ts)*lac*::Tn*3* plasmid had transferred, only 2 of 16 of these colonies tested (5022-1-C-1 and 5022-1-C-9) were found to agglutinate in form I antisera, i.e., had coinherited the form I trait.

Transconjugant strain 5022-1-C-9 (Lac$^+$ Met$^-$ Nal^r), which had inherited form I antigen expression, was used subsequently as a donor in conjugal matings with various recipient strains including *S. sonnei* form II strains 53G and 775-14, *S. flexneri* 2a strain 2457 G1, and *Salmonella*

typhi strain 643 Holland C⁻. A high proportion of the Lac⁺ transconjugants resulting from these matings expressed the form I antigen. Form I-positive *S. flexneri* 2a and *Salmonella typhi* cells also retained their ability to agglutinate in their parental typing antisera; i.e., they agglutinated in *S. sonnei* form I antisera and in either *S. flexneri* type 2 or *Salmonella* 9,12 antiserum. Further studies on one form I-expressing *S. flexneri* 2a strain (5037-2-8) showed, as expected, that these cells are capable of adsorbing all of the form I antibody from form I antiserum and, when injected into rabbits, produce form I antibodies in addition to *S. flexneri* 2a antibodies. Moreover, these form I *S. sonnei*, *S. flexneri*, and *Salmonella typhi* strains were capable of transferring the form I antigen back to form II *S. sonnei*. The form I plasmid is unstable and is readily lost from both *S. flexneri* and *Salmonella typhi* transconjugants at a frequency comparable to that seen in *S. sonnei*.

Plasmid analyses of donor, recipient, and transconjugant cells. To compare plasmid content, plasmid DNA was extracted from a variety of donor, recipient, and transconjugant cells and analyzed by agarose gel electrophoresis (Fig. 2). Virulent 53G cells carry two plasmids, the large 120-Mdal form I plasmid and a small cryptic plasmid of about 2 Mdal which is not always observed in gels (see Fig. 1 legend and Fig. 2A). In addition to harboring these plasmids, the Lac⁺ derivative of 53G (strain 5006-7-3) contains a plasmid of the size expected for the F′(Ts)*lac*::Tn*3* element (~80 Mdal) and a smaller plasmid of ~5 Mdal (Fig. 2B). This 5-Mdal plasmid is a cryptic plasmid, normally found in strain HU679, that was cotransferred with F′(Ts)*lac*::Tn*3* to 5006-7-3 (unpublished data). As shown in Fig. 1 and verified in Fig. 2C and E, form II derivatives of 53G have lost the 120-Mdal plasmid. Strain 5022-1-C-9 is the transconjugant constructed by a conjugal mating between 5006-7-3 (Fig. 2B) and 772-7 II (Fig. 2C). The 5022-1-C-9 transconjugant contains three large plasmid DNA species, only two of which are visible in Fig. 2D. The largest molecular species, estimated to be 200 Mdal in size, apparently represents a recombinant between the form I plasmid and the F′(Ts)*lac*::Tn*3* element, whereas a faint band representing the F′(Ts)*lac*::Tn*3* plasmid is also visible. Apparently, the large composite plasmid dissociates into its component replicons in the transconjugants. As a result, DNA bands representing the composite and the two individual component plasmids are often observed when the plasmid

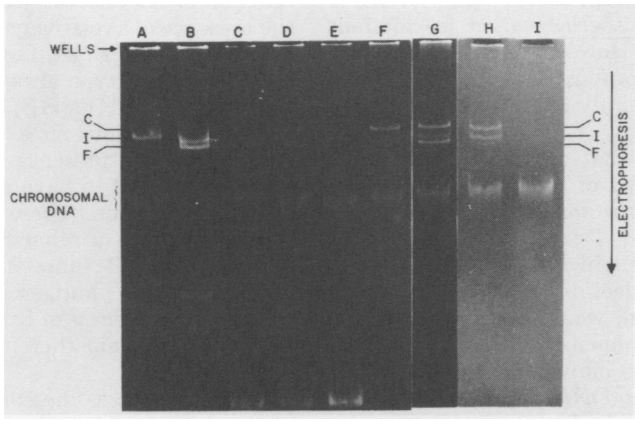

FIG. 2. *Agarose gel electrophoretic profiles of circular plasmid DNA, obtained by the method of Hansen and Olsen (5), from selected donor and recipient cells, as well as from transconjugant strains that have received the form I trait. See the text for DNA isolation and gel electrophoresis procedures. Plasmid profiles of: (A) form I S. sonnei strain 53G; (B) donor strain 5006-7-3, a form I 53G strain that harbors the F′(Ts)-lac::Tn3 plasmid; (C) recipient S. sonnei strain 772-7 II; (D) transconjugant S. sonnei strain 5022-1-C-9, made by mating strains whose profiles are shown in wells B and C; (E) recipient form II 53G strain; (F) transconjugant S. sonnei strain 5059-1-2, made by transferring the form I trait from 5022-1-C-9 to 53G form II cells; (G) S. sonnei strain 5059-1-2, but a different DNA preparation from that shown in F; (H) transconjugant Salmonella typhi 643 Holland C⁻ strain, which had received the form I trait during conjugal mating with strain 5022-1-C-9; (I) plasmid-free, S. typhi 643 Holland C⁻ strain. Strain 5006-7-3, shown in well B, harbors an additional small cryptic plasmid which cotransferred from strain HU679. The small plasmid seen in well D was incidentally coinherited from donor strain 5006-7-3. However, similar small plasmids were not observed in most transconjugants (e.g., wells F and G). The gel positions of fragmented chromosomal DNA, F′(Ts) lac::Tn3 plasmid DNA (labeled F), form I plasmid DNA (labeled I), and composite plasmid DNA (labeled C) formed by recombination between a form I and an F′(Ts)lac::Tn3 molecule are indicated.*

profiles are examined. The migration positions of these three plasmid species are indicated on each side of Fig. 2. Because of differences in DNA concentration or the volume of sample added to a gel, corresponding DNA species do not always migrate identically. For example, on careful examination the form I plasmid band in well A (Fig. 2) migrated slightly more slowly than the corresponding form I band in well B. However, from analyzing many different DNA preparations of these strains on different gels, we feel that the following overall interpretation is fair. The 5022-1-C-9 transconjugant strain was able subsequently to donate the form I trait to other recipients including *S. sonnei* form II (Fig. 2E, F, G), *S. flexneri*, and *Salmonella typhi* (Fig. 2H, I). In all cases examined, the transconjugants for the form I plasmid were observed to have received a large composite molecule which dissociated to its individual components. The proportions of each component plasmid relative to the composite molecule varied for each DNA preparation. For instance, the DNA profiles of two different DNA preparations of the same *S. sonnei* transconjugant strain are shown in Fig. 2F and G. It is important to emphasize that DNA preparations obtained from strain 5022-1-C-9 and all other transconjugants for the form I trait gave plasmid profiles identical to that shown in Fig. 2G, except that the proportions of the three plasmid species varied in different DNA preparations. However, in many transconjugant DNA preparations, one or more of these DNA bands were faintly visible or invisible (see Fig. 2D, F, H).

It would appear that the form I plasmid is cotransferred as part of a composite molecule consisting of an entire form I::Tn*3* plasmid recombined with the F′(Ts)*lac*::Tn*3* plasmid. This composite structure, which should contain internal regions of nucleotide sequence homology (i.e., the Tn*3* units), would be expected to be unstable in a recombinationally proficient host and to separate into individual components. In fact, transconjugant DNA profiles display the expected result; i.e., both the composite molecule and component plasmids are observed (see Fig. 2G and H). Finally, although various other mating systems have been attempted, transfer of the nonrecombined form I plasmid has not been observed.

Virulence studies. Form I cells of three of the four wild-type *S. sonnei* strains (i.e., 53G, 50E, and MBI) included in this study were found to be virulent as assessed by the Sereny test (12). Form II variants of these three virulent strains were, as expected, uniformly avirulent. However, strain 9774, though expressing the form I antigen, was avirulent and apparently lacks some other requisite of virulence.

The presence of RP4 or a wild-type F*lac* plasmid in form I 53G cells did not affect the virulence of these host bacteria. Quite unexpectedly, however, form I 53G cells carrying the F′(Ts)*lac*::Tn*3* plasmid or the parental F′(Ts)*lac* plasmid were always avirulent. It appears that some trait on this F′(Ts)*lac* plasmid impairs the virulence of *S. sonnei* cells.

DISCUSSION

In this study, four representative *S. sonnei* strains isolated from widely separated geographical regions of the world were examined (Table 1). Under low-power stereomicroscopy, even-edged, form I *S. sonnei* colonies, when restreaked on solid media, were observed to undergo a rapid and irreversible colonial morphology transition to rough-appearing, uneven-edged colonies, termed form II. Form I and II cell types were verified serologically in properly prepared antisera. This shift from form I to form II has previously been reported to be due to the loss of an immunodeterminant group containing 2-amino-deoxy-L-altruonic acid (6) from the somatic antigen of form I cells, and always results in the loss of virulence.

Electrophoretic analysis of the plasmid DNA of form I and form II variants of four isogenic sets of representative *S. sonnei* strains revealed the presence of a relatively large plasmid in each form I strain (Fig. 1A, C, E, G). Transition to the form II cell type always resulted in loss of the large plasmid (Fig. 1B, D, F, H). Other small plasmids of various sizes are present in some strains, but the presence or absence of these plasmids seemed to have no bearing on form I antigen expression. These data, together with similar analyses of other *S. sonnei* strains not listed in Table 1 (unpublished data), demonstrate that form I antigen synthesis and, consequently, invasiveness of *S. sonnei* are controlled by a large plasmid (herein termed the form I plasmid).

Studies to assess the conjugal transferability of the form I antigen trait were subsequently conducted. No easily selectable characteristic could be identified on the large, presumably self-transmissible plasmids responsible for form I antigen synthesis. Although we have evidence only for the lack of expression by the form I plasmid of six antibiotic resistance traits and four metabolic characters, M. Popoff and P. Sansonetti of the Institut Pasteur, Paris, have tested more than 150 characters, including all commonly employed antibiotics, and find no correlation between the presence of the form I plasmid and expression of any of these traits (personal communication). Therefore, potential

transconjugants for the form I antigen trait had to be detected visually and serologically. Self-transfer of the nonrecombined form I plasmid from *S. sonnei* donors to form II recipients was not detected after conjugal mating experiments carried out at either 25, 32, or 37°C for 24 h in broth or on a solid medium. Mobilizing plasmids such as F*lac* or RP4 in form I cells also did not effect transfer of the form I trait at a detectable frequency. In the continuing assumption that the form I plasmid is conjugally self-transmissible, the ampicillin resistance transposon (Tn*3*) was transposed to it from the F′(Ts)*lac*::Tn*3* plasmid. Since the Tn*3* transposon appears to transpose to plasmids more often than to the bacterial chromosome (8), it seemed probable that the form I plasmid would be labeled with Tn*3* in most strains that had received this transposon. The Ap^r character could then be used to detect low-frequency plasmid transfer. However, of more than 50 strains examined that had retained Ap^r after loss of the F′(Ts)*lac*::Tn*3* plasmid after growth at 42°C, none was observed to transfer either Ap^r or the form I antigen trait to appropriate recipient cells. Collectively, these data indicate that the form I plasmid is not self-transmissible, at least in the temperature range (25 to 37°C) that is optimal for most conjugative plasmids.

It seemed likely, however, that the form I plasmid had received the Tn*3* element in at least some cases. We reasoned that recombination between the Tn*3* unit on the form I plasmid and Tn*3* on the F′(Ts)*lac*::Tn*3* plasmid would generate a composite molecule that might transfer form I antigen-synthesizing ability. A form I *S. sonnei* strain (5006-7-3), to which Tn*3* had been transposed and which had subsequently received the F′(Ts)*lac*::Tn*3* plasmid, was observed to transfer the form I antigen trait to recipient form II cells. Plasmid analyses of donor, recipient, and transconjugant strains indicated that the F′(Ts)*lac*::Tn*3* plasmid had recombined with the form I plasmid, presumably via the homologous Tn*3* segments in each plasmid. As a consequence, a large composite molecule apparently consisting of both entire parental plasmids was generated (Fig. 2). Conjugal transfer of the form I trait seemingly resulted from transfer of the large composite molecule. The composite plasmid, due to intramolecular areas of nucleotide sequence homology (i.e., Tn*3* units), was expected to be unstable in a recombinationally proficient host. In fact, in transconjugant cells that had received the form I antigen trait, the composite plasmid was observed to dissociate into plasmids which appeared identical in size to the parental form I and F′(Ts)*lac*::Tn*3* plasmids (Fig. 2D, F, G, H). These molecular and genetic data support the above interpretation that form I antigen synthesis is controlled by a relatively large, non-self-transmissible plasmid (~120 Mdal in strains 53G, 50E, and MBI) in *S. sonnei*. In addition, these results demonstrate that upon reintroduction of the form I plasmid into form II recipient cells, the form I cell surface antigen is expressed. Strain 5006-7-3, as well as various *S. sonnei* transconjugants for form I antigen-synthesizing ability, was able to donate the composite plasmid to other form II *S. sonnei*, to *S. flexneri* 2a strain 2457 G1, and to *Salmonella typhi* strain 643 Holland C^-. The finding that form I antigen is expressed in a non-*Shigella* host strongly suggests that the structural determinants for the form I antigen, and not just regulatory genes, are located on the form I plasmid.

Although we were able to transfer the form I antigen to form II cells, we have not yet been successful in reestablishing the ability to penetrate epithelial cells. The form I antigen is no doubt a necessary requisite for virulence, but it cannot be the only factor, since form I^+ *S. sonnei* strains exist which are avirulent. Strain 9774, included in this study, is such an example. The introduction of RP4 or one of several wild-type F*lac* plasmids into virulent form I strains did not affect their ability to invade epithelial cells. However, the ability to invade epithelial cells, but not form I antigen expression, was lost in cells carrying the F′(Ts)*lac*::Tn*3* plasmid, the mobilizing factor used to construct the form I donor strain. Thus, although the F′(Ts)*lac*::Tn*3* plasmid was necessary to obtain transfer of the form I plasmid, some property on the former molecule is responsible for causing loss of virulence. Though speculative, several loci involved in cell wall synthesis which are located close to the *lac* region in the *E. coli* chromosome may be on the F′(Ts)*lac*::Tn*3* plasmid. Synthesis of some *E. coli* cell wall component in *Shigella* may somehow result in avirulence. Further studies to reestablish virulence, in addition to form I antigen expression, in form II cells are presently being conducted.

We reemphasize the fact that most form I *S. sonnei* strains readily undergo the transition to form II colonies. Though not mentioned above, the colonial morphology transition for strain 53G at 25°C did not differ in frequency from that observed at 37°C. Therefore, this form I plasmid is very unstable in *S. sonnei* at temperatures that would normally be encountered in nature. The high rate of form I plasmid loss in *S. sonnei* in the absence of selective pressure would appear, however, to be evolutionarily disadvantageous for this bacterium. Despite these findings, *S. sonnei* continue to be the major cause of

shigellosis in the United States. These observations suggest that the form I antigen or some other plasmid-encoded trait is specifically selected for in the human intestine, which would maintain *S. sonnei* cells in the virulent state. Alternatively, *S. sonnei* might continuously reinherit the form I plasmid from some other bacterial host, presumably in the human alimentary tract. Further experiments to define the intermolecular relationships among the form I plasmids and molecular cloning of the plasmid-borne virulence determinants are currently under way.

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ADDENDUM

On submitting this manuscript, we became aware that P. Sansonnetti of the Institut Pasteur, Paris, has independently conducted similar studies and has concluded that form I antigen synthesis is plasmid controlled.

LITERATURE CITED

1. **Davis, B. D., and E. S. Mingioli.** 1950. Mutants of *Escherichia coli* requiring methionine or vitamin B_{12}. J. Bacteriol. **60**:17-28.
2. **Ewing, W. H.** 1972. Isolation and identification of *Salmonella* and *Shigella*, p. 45. Publication no. 76-8098. Center for Disease Control, Atlanta, Ga.
3. **Gemski, P., and S. B. Formal.** 1975. Shigellosis: an invasive infection of the gastrointestinal tract, p. 165-169. *In* D. Schlessinger (ed.), Microbiology—1975. American Society for Microbiology, Washington, D.C.
4. **Gemski, P., A. Takeuchi, O. Washington, and S. B. Formal.** 1972. Shigellosis due to *Shigella dysenteriae* 1: relative importance of mucosal invasion versus toxin production in pathogenesis. J. Infect. Dis. **5**:523-530.
5. **Hansen, J. B., and R. H. Olsen.** 1978. Isolation of large bacterial plasmids and characterization of the P2 incompatibility group plasmids pMG1 and pMG5. J. Bacteriol. **135**:227-238.
6. **Kontrohr, T.** 1977. The identification of 2-amino-2-deoxy-L-altruonic acid as a constituent of *Shigella sonnei* phase I lipopolysaccharide. Carbohydrate Res. **58**: 498-500.
7. **Kopecko, D. J., J. Holcombe, and S. B. Formal.** 1979. Molecular characterization of plasmids from virulent and spontaneously occurring avirulent colonial variants of *Shigella flexneri*. Infect. Immun. **24**:580-582.
8. **Kretschmer, P. J., and S. N. Cohen.** 1977. Selected translocation of plasmid genes: frequency and regional specificity of translocation of the Tn*3* element. J. Bacteriol. **130**:888-899.
9. **LaBrec, E. H., H. Schneider, T. J. Magnani, and S. B. Formal.** 1964. Epithelial cell penetration as an essential step in the pathogenesis of bacillary dysentery. J. Bacteriol. **88**:1503-1518.
10. **Macrina, F. L., D. J. Kopecko, K. R. Jones, D. J. Ayers, and S. M. McCowen.** 1978. A multiple plasmid-containing *Escherichia coli* strain: convenient source of size reference plasmid molecules. Plasmid **1**:417-420.
11. **Meyers, J. A., D. Sanchez, L. P. Elwell, and S. Falkow.** 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. **127**:1529-1537.
12. **Sereny, B.** 1955. Experimental *Shigella* conjunctivitis. Acta Microbiol. Acad. Sci. Hung. **2**:293-296.