The Shufflon of *Salmonella enterica* Serovar Typhi Regulates Type IVB Pilus-Mediated Bacterial Self-Association

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Previously, it was shown that type IVB pili encoded by the *Salmonella enterica* serovar Typhi *pil* operon are used to facilitate bacterial entry into human intestinal epithelial cells in vitro and that such entry is inhibited by purified prepilin (pre-PilS) protein (X.-L. Zhang, I. S. M. Tsui, C. M. C. Yip, A. W. Y. Fung, D. K.-H. Wong, X. Dai, Y. Yang, J. Hackett, and C. Morris, Infect. Immun. 68:3067–3073, 2000). The *pil* operon concludes with a simple shufflon, and a recombinase gene product (Rci) inverts DNA in the C-terminal region of the *pilV* gene to allow synthesis of two distinct PilV proteins, PilV1 and PilV2, which are presumptive minor pilus proteins. We show here that the type IVB pili mediate bacterial self-association, but only when the PilV1 and PilV2 proteins are not expressed. This may be achieved in wild-type serovar Typhi by rapid DNA inversion activity of the shufflon. We show that the inversion activity inhibits the expression of genes inserted between the 19-bp inverted repeats used for Rci-mediated recombination and that the activity of Rci increases when DNA is supercoiled. The data suggest that serovar Typhi self-associates under conditions (such as low oxygen tension in the gut) that favor DNA supercoiling. These results explain (i) the function of the serovar Typhi shufflon and (ii) why there are only two possible shufflon states, in contrast to the many possible states of other shufflon systems. The data further indicate that a very early step in serovar Typhi pathogenesis may be type IVB pilus-mediated self-association of bacteria in the anaerobic human small intestine prior to invasion of the human gut epithelium. The suggested type IVB pilus-dependent step in typhoid fever pathogenesis may partially explain the enhanced invasiveness of serovar Typhi for humans.

The type IVB pilus operon of *Salmonella enterica* serovar Typhi contains a *pilS* gene encoding the structural pilin (3, 15). A *pilS* mutant of serovar Typhi exhibited much reduced adhesion to and invasion of human epithelial gastrointestinal cells in vitro, and soluble purified pre-PilS protein (retaining the signal sequence normally cleaved when the protein is excreted to form insoluble pili based on polymerized PilS) inhibited bacterial invasion (15). These data indicated that the type IVB pili might have an important function in the pathogenesis of serovar Typhi in humans.

The serovar Typhi *pil* operon concludes with a simple shufflon, and the Rci gene product, encoded by the *rci* gene adjacent to the *pil* operon, acts to invert DNA between a pair of 19-bp inverted repeats to place one of two possible C termini on the *pilV* gene (14). No function in pathogenesis or otherwise has yet been assigned to the serovar Typhi shufflon. It is notable that the serovar Typhi shufflon is simple compared to the shufflons which terminate the *pil* operon of plasmids R64 and R721. The latter shufflons have seven and six 19-bp repeats, respectively, to form seven or six different *pilV* genes by Rci-mediated recombination. In these plasmids, the various PilV proteins selectively recognize determinants on the exterior of enterobacterial species, facilitating transfer of the conjugative plasmids in liquid mating (2, 4, 12). There is no suggestion that serovar Typhi is a donor (even of the Hfr type) in mating, and the serovar Typhi genome sequence lacks the *tra* gene complement which would be expected if the bacterium were capable of DNA transfer (9).

It is recognized that the bundle-forming type IVB pili of enterotoxigenic *Escherichia coli* act to increase bacterial virulence by promotion of bacterial aggregation (1), and it is thought that the type IVB pili of *Vibrio cholerae* (essential, in the infant mouse model, for the development of choleraic disease) may have a similar function (6). It was of interest, then, to explore possible functions of the shufflon in typhoid fever pathogenesis. In particular, we wished to ask if the shufflon of serovar Typhi might somehow be involved in the promotion of bacterial self-association by the type IVB pilus, which in turn could enhance bacterial invasion of the cells of the human small intestinal wall.

**MATERIALS AND METHODS**

**Materials.** Reagents were of molecular biology grade. Enzymes active on DNA were obtained from either Invitrogen or Roche and were used as directed by the suppliers. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from Amersham. Bio-Rad was the supplier of polyvinylidene difluoride membranes.

**Media.** Luria-Bertani broth (LB) was prepared as described by Miller (8). Solid medium contained 1.5% (wt/vol) agar. Antibiotics were added, when appropriate, at the following concentrations: tetracycline, 5 to 15 μg/ml; nalidixic acid, 15 μg/ml; chloramphenicol, 30 μg/ml; streptomycin, 50 μg/ml; kanamycin, 50 μg/ml; and ampicillin, 100 μg/ml.

**Strains and cassettes.** Serovar Typhi J341 (Ty2 Vi−) (15) is the wild-type strain that was used in this work. *S. enterica* serovar Typhimurium J357 [Δ*rrtD ivs452 metE551 lys metA22 hsdM k1 mcrA Kmr Tcr rKm Tci] cured of virulence plasmid pSLT served as a modifying strain prior to transformation of recombinant plasmids to serovar Typhi. To make serovar Typhi strains proficient in transfer of a conjugative plasmid, an *Escherichia coli* K-12 strain carrying the conjugative plasmid pSU760 (Rex::Tn1535) (Km’ Te’) (11) was conjugated with spontaneous Rif’ mutants of serovar Typhi (ca. 106 bacteria of both strains) on solid medium for 1 h at 30°C, and transconjugants

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were obtained and purified by subsequent plating on solid medium with tetracycline and rifampin, also at 30°C. PCR checks with primers based on R64 pil and tra sequences confirmed that such DNA was absent from R611; transfer of such a plasmid in liquid mating, therefore, does not involve an R64-like type IVB pil operon.

For use as recipients in liquid mating experiments, spontaneous pils were selected in one of the mutants of serovar Typhi strains were obtained by plating ca. 3 × 10^8 bacteria on plates with streptomycin (50 µg/ml). Mutants were obtained at a frequency of ca. 10^{-9}. E. coli K-12 strain DH5α [supE44 ΔlacU169 (Δ801 lacZAM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1] was the usual host for recombinant plasmids. E. coli JM109 [F’ proA1348 dcm ΔlacZΔM15 proA+ B’ +c14 (McaI) Δlac-proAB thi-glu-962 endA1 hisD49 recA1 hsdR17 (rK-, mK-) Δ28A1 supE44 lacI159] was used as the host for plasmids pUST151 to pUST156. A Tc r cassette used in plasmid construction (inserted between the pilV' 19 bp repeats) was amplified by PCR from pBR322. The xylE gene used in similar tests was obtained by PCR from pCM20 (7).

Growth of bacteria with tetracycline. E. coli JM109-based strains containing single plasmids belonging to the pUST151-pUST153 series were grown for 16 h at 37°C in 5 ml of LB with ampicillin. Cultures (0.4 ml) were inoculated in triplicate into 40 ml of prewarmed LB containing tetracycline (0.01 mM) and IPTG (0.1 mM) with or without nalidixic acid. After the initial optical density at 600 nm (OD_{600}) was determined, cells were grown with shaking at 37°C, and aliquots were removed at subsequent time points up to 9 h. Three separate OD_{600} values were averaged and plotted. The tests were repeated three times.

Measurement of XylE activity. E. coli JM109-based strains containing single plasmids belonging to the pUST154-pUST156 series were grown for 16 h at 37°C in 5 ml of LB with ampicillin and IPTG (0.1 mM). Some 3-ml portions of cultures (at OD_{600} values of ca. 1.5) were pelleted, washed with 20 mM phosphate buffer (pH 7.2), and resuspended in 100 mM phosphate buffer (pH 7.5) with 10% (vol/vol) acetone for sonication. Cells were disrupted with a Branson 450 Sonicator (50% pulsed operation, output control 4) for 2 min. Each clear lysate was centrifuged at 20,000 × g for 15 min at 4°C, and the cell extract was collected and used for a catechol 2,3-dioxxygenase assay. Catechol was obtained from Aldrich Inc., Milwaukee, Wis. Enzyme activity was expressed in pico moles of 2-hydroxymuconic semialdehyde produced per minute per microgram of protein.

Analytical methods. For plasmid preparation, restriction enzyme digestion, and other DNA manipulations we used the methods of Sambrook et al. (10). For development of immunoblots we used the Enhanced Chemiluminescence system (Amersham). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was usually performed with a 5% (wt/vol) polyacrylamide stacking gel and an 18% (wt/vol) polyacrylamide separating gel.

Terminology used in describing DNA sequences in the pilV region. In wild-type serovar Typhi, two possible orientations of the pilV C-terminal region are possible, and one is predominating (14). In this orientation, the N-terminal region of the pilV gene is followed by the 19-bp sequence 5′-GTGCCAATCCGGATTTG-GG-3′, and this sequence is referred to as the V1 sequence below. After the DNA which includes the C-terminal portion of the pilV gene is the sequence 5′-CCGGATCCGGATGACG-3′, which is also 19 bp long, and this sequence is referred to as the V2 sequence below (the base which differs from the base in the reverse complement of the V1 sequence is underlined). These two 19-bp sequences are used by Rci to invert intervening DNA. The two V1 orientation indicates that a promoter external to the invertible DNA reads first through the V1 sequence, across invertible DNA, and out through the V2 sequence. In this study, the promoter in question was the lac promoter on a plasmid bearing cloned DNA from the shufflon. When the V2 orientation is used, the sequence is promoter-V2 sequence-invertible DNA-V1 sequence.

Construction of serovar Typhi pil mutants. The mutations used in this work are shown in Fig. 1A. In general, a pil mutation was constructed in a plasmid bearing part of the pil operon DNA, and it was accompanied by introduction of a selectable antibiotic resistance marker. The modified plasmids were transferred to serovar Typhi via the S. enterica serovar Typhimurium modifying strain JS37, with selection for both the vector-encoded antibiotic resistance and the antibiotic resistance introduced into the pil DNA. As previously described (15), growth at 42°C destabilizes plasmids, and recombination of the plasmid-borne mutations into the serovar Typhi chromosome could be readily selected.

To construct the ΔpilV mutation, plasmid pUST108 (14), carrying intact pilV' and rci genes, was cut with BglII and a 1,582-bp fragment lacking the C-terminal 895 bp of pilV' and the N-terminal 477 bp of rci (GenBank accession no. AF000001) was ligated with the pUC4K Km'^+ cassette. Construction (in plasmids) of the constitutive pilV' and pilV' mutations has been described previously (14). In serovar Typhi mutants carrying these mutations the rci gene is damaged so that the PilV2 protein is not made, but not both, is made. Construction of the pilV mutation with the rci promoter and an upstream Cm’ cassette, both inserted in a noncoding region between the pilV and pilN genes, in which the rci promoter drives the transcription of the pilN-pilV genes, has been described previously (15).

FIG. 1. (A) Mutations in the pil operon of serovar Typhi. The wild-type (wt) pil gene arrangement (15) is shown at the top. Strain construction details are described in Materials and Methods. The pilV' mutation with the pilN-pilV' intergenic region (Cmr' strain) has been described previously (15); when present, the tac promoter drives the pilN-pilV' genes. Insertion of a tac promoter into the pilM-pilN intergenic region (Cmr' strain) has been described previously (15); when present, the tac promoter drives the pilN-pilV' genes and greatly increases the expressed level of external PilS protein (15). (B) PCR showing the orientation of the pilV gene. In the wild-type strain, primers detecting either the pilV1 orientation (primer pair 1) or the pilV2 orientation (primer pair 2) gave PCR bands, but only one set of primers yielded PCR products when the pilV' gene was fixed in one of the two possible orientations (14). Insertion of a tac promoter into the pilM-pilN intergenic region (Cmr strain) has been described previously (15); when present, the tac promoter drives the pilN-pilV genes.

To amplify pilV' genes (Fig. 1B), three primers were used. The sequence of primer prp/pilV5' was 5′-GGATTGGATACGATGACGATC3′, and this primer hybridizes to DNA (nucleotides 16847 to 16876 in GenBank...
accession no. AF000001) at the beginning of pilV; the primer has a modified pilV sequence, which creates a BamHI site that was used in previous cloning work. The sequences of primers \( \text{pilPilV}^{13} \) and \( \text{pilPilV}^{23} \) were 5'-CAGGGCATGGAATCAGAGGCAATG-3', respectively (both were modified from the pilV sequence to include EcoRI sites), and these primers hybridize to DNA invertible by Rci lying beyond the end of the pilV gene. As implied, these primers act with \( \text{pilPilV}^{35} \) to amplify only pilV1 or pilV2 DNA. The fragment sizes expected are 1,334 and 1,312 bp, respectively.

Preparation of pili. Various Cmr strains were grown in shaking (200 rpm) cultures for 14 h at 30°C, and the concentrations were each adjusted to an OD600 of 0.5 with sterile medium. The cultures (250 ml) were centrifuged at 9,200 \( \times g \) for 10 min, and the supernatants were then centrifuged at 140,000 \( \times g \) for 1 h. The pellets were resuspended in 0.2 ml of phosphate-buffered saline and contained type IVB pili; the yield was ca. 10% of that obtained by sonication of concentrated bacteria (15).

Experiments with human epithelial gastrointestinal cells. Human embryonic intestine cell line INT407 was obtained from the American Type Culture Collection and was used in bacterial association tests with serovar Typhi strains as previously described (15). To obtain relative measures (compared to wild-type serovar Typhi) of INT407 cell entry by the \( \Delta \text{pilV} \), pilV1c, and pilV2c mutants, wild-type serovar Typhi and the Km' serovar Typhi mutant studied were mixed (pairwise) in equal numbers before addition to the INT407 cells. After killing (with gentamicin) of bacteria which did not enter the INT407 cells, residual bacteria were enumerated on solid media with and without kanamycin to obtain values for INT407 cell entry for mutant and wild-type strains, respectively.

Liquid and plate mating tests. Donor strains (serovar Typhi strains carrying a conjugative Te' plasmid and Str') and recipients (various Str' serovar Typhi strains) were grown with aeration for 16 h at 30°C in the presence of antibiotics (either tetracycline or streptomycin as appropriate). Donors were then subcultured in LB with tetracycline and grown with aeration at 30°C for 4 h to the log phase. The recipient cultures were washed, and the OD600 values were adjusted to 0.5 with sterile medium. The donor cells were also washed and resuspended at a concentration of ca. 5 \( \times 10^8 \) cells/ml. Equal volumes (0.8 ml) of donors and recipients were mixed, and liquid mating mixtures were incubated for 1.5 h at 30°C. Dilutions of the mating mixtures were plated with tetracycline (selecting for the R-factor) and streptomycin (counterselecting for the donors), and colonies were enumerated on LB plates with tetracycline (5 \( \mu \)g/ml) and streptomycin (50 \( \mu \)g/ml) after incubation for 20 h at 30°C. For plate mating, donors and recipients were mixed at a ratio of 1:9 (a total of ca. 109 bacteria), and 80-\( \mu \)l portions of the mixtures were spotted on a dry antibiotic-free plate. After incubation for 1.5 h at 30°C, the area exhibiting bacterial growth was carefully scraped into sterile liquid medium (1 ml), and dilutions of the suspension were plated for enumeration of transconjugants.

RESULTS AND DISCUSSION

PilV proteins are not required for PilS expression or for INT407 cell invasion. Serovar Typhi type IVB pili may contain small amounts of the two possible PilV proteins (12), which, in R64, are thought to pilus tip adhesins (4). In R64-bearing E. coli K-12 strains, PilV proteins appear to be required for efficient pilus synthesis, as only a trace of extracellular PilV protein was seen when the pilV gene was inactivated (13). The PilV proteins may not be required for PilS production in serovar Typhi, however. When the tac promoter drives the pilN-pilV genes in otherwise wild-type serovar Typhi and in a \( \Delta \text{pilV} \) mutant, the levels of type IVB pili produced appear to be comparable (Fig. 1C). It was necessary to use expression of tac-driven pil genes for the immunoblot analyses, as the level of PilS expression in wild-type serovar Typhi is very low (15). For clarity, it should be emphasized that serovar Typhi strains with some pil genes driven by the tac promoter were used only to obtain material for the immunoblots shown in Fig. 1C; such strains were not used elsewhere in this work. The INT407 cell entry levels of wild-type serovar Typhi, a pilS mutant (15), and the other mutants (\( \Delta \text{pilV} \), pilV1c, and pilV2c) shown in Fig. 1 were measured. As previously noted, the level of entry of the serovar Typhi pilS mutant was much reduced compared to the level of entry of wild-type bacteria, but the levels of INT407 cell entry of the \( \Delta \text{pilV} \), pilV1c, and pilV2c strains did not differ significantly from the level exhibited by the wild-type organism (data not shown), as might be expected in light of the approximately equal amounts of PilS protein expressed by all four strains (Fig. 1C).

PilV proteins inhibit self-association of serovar Typhi bacteria. A clue to a possible function of the PilV proteins and of the shufflon which inverts DNA in the C-terminal region of the pilV gene was provided by the observation that a serovar Typhi \( \Delta \text{pilV} \) mutant (but not wild-type, pilS, pilV1c, or pilV2c strains) formed granular bacterial pellets after growth (without shaking) at 37°C for 16 h (data not shown). In efforts to quantitate the bacterial self-association, a liquid mating assay was developed. The rationale was that self-associating bacterial strains, acting as recipients in liquid mating, should tend to enmesh some donor bacteria if the recipients are in 10-fold numerical excess with respect to the donor cells. This entrapment of donors in a developing bacterial pellet should be reflected in a higher level of transfer of a conjugative plasmid from donor to recipient than would be the case if the recipient strains did not self-associate. To develop the assay, five donors (wild-type serovar Typhi and the pilS, \( \Delta \text{pilV} \), pilV1c, and pilV2c strains), all carrying the conjugative plasmid pRU670, were prepared, and spontaneous rpsL (Str') mutants of the same five strains that were plasmid free served as recipients. A total of 25 distinct crosses were thus possible. Application of an assay of this type (Fig. 2) showed that the \( \Delta \text{pilV} \) mutant strain was indeed ap-
and plasmids and a test plasmid (pUST156) were constructed. The cas-
19-bp repeats. Positive (pUST154) and negative (pUST155) control

FIG. 3. Details of two series of cassettes inserted into pUC18-
based plasmids to test the effect of Rci-mediated inversion of DNA
between 19-bp repeats on transcription of genes located within the
repeats. (A) Plasmids pUST151 to pUST153 carry the Te' gene be-
tween the 19-bp repeats. In the positive control plasmid, pUST151, the
Te' gene is in the V1 orientation and may be transcribed from the
lac promoter. In the negative control plasmid, pUST152, the Te' gene is in
the V2 orientation, and transcription of this gene from the lac prom-
eter is not possible. The test plasmid, pUST153, carries an intact rci
gene, and DNA including the entire Te' gene may be inverted.

approximately threefold more effective in terms of recipient abil-
ity than the pilS, pilV1c, and pilV2c strains were. The wild-type
strain showed intermediate and variable recipient ability, and
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Serovar Typhi shuffl on regulates transcription of the pilV
gene. It was thought likely that Rci-mediated inversion of DNA
between the 19-bp repeats might terminate contemporaneous
transcription events if the RNA polymerase were located on
the inverted DNA segment. To test this idea, reporter genes
coding an easily measurable phenotype (bacterial growth in
the presence of nalidixic acid [see below]), densitometer scans of plasmid restric-
tion digests showed that ca. 95% of the plasmid was in the V1
orientation permissive for Tcr gene transcription

FIG. 4. Gel electrophoresis of plasmid restriction digests, showing
that the equilibrium levels of DNA in the V1 and V2 orientations differ
in two plasmids containing DNA invertible by Rci. Lane A contained
a SacI digest of pUST153; DNA bands from the V1 orientation are
well-separated, but the sizes of the DNA bands from the V2 orienta-
tion are similar to the sizes of the bands from the V1 orientation and
cannot be seen easily. Lane B contained an EcoRV digest of pUST153;
two DNA bands from the V1 orientation are poorly separated, but two
DNA bands from the V2 orientation are different sizes and may be
faintly seen (arrows). A densitometer scan showed that ca. 95% of
the plasmid was in the V1 orientation permissive for Te' gene transcription
from the lac promoter. Lane C contained molecular size control stan-
dards. Lane D contained a HindIII digest of pUST156; DNA bands
from both the V1 and V2 orientations are well separated (bands from
the V2 orientation are indicated by arrows). A densitometer scan
showed that ca. 40% of the plasmid was in the V2 orientation permiss-
ive for xylE transcription from the lac promoter.

the lac promoter. In the negative control plasmid, pUST152,
the Te' gene is in the V2 orientation, and transcription from the
lac promoter is not possible. The test plasmid, pUST153,
carries an intact rci gene, and DNA including the entire Te' gene
may be inverted. In the second series of plasmids (pUST154 to pUST156) (Fig. 3B), a promoterless xylE gene
was placed between the 19-bp repeats. In this plasmid series
(which includes the positive control plasmid pUST154 and the
negative control plasmid pUST155), the V2 orientation in plas-
mid pUST156 is permissive for transcription of the xylE gene
from the lac promoter.

Plasmids pUST153 and pUST156 both contain Rci-invert-
ible DNA. Preparations of either plasmid, therefore, contain
two distinct DNA molecules, reflecting the presence of the two
distinct Te' (pUST153) or xylE (pUST156) gene orientations.
The use of restriction enzymes which cut asymmetrically within
Rci-invertible DNA allowed estimates to be made of the propor-
tions of the rciI+ plasmid samples in the two possible ori-
entations. In pUST153, the V1 orientation (which is permissive
for transcription of the Te' gene from the lac promoter) was
heavily favored (Fig. 4, lanes A and B). Under all of the growth
conditions examined (including growth in the presence of nal-
idixic acid [see below]), densitometer scans of plasmid restric-
tion digests showed that ca. 95% of the plasmid was in the V1
orientation. It is important to recognize that this reflects only
the equilibrium levels of the two possible orientations. The
rates of inversion between orientations (which may differ with different growth conditions [see below]) are not determined by such methods. With pUST156, however, densitometer scans of plasmid restriction digests showed that ca. 40% of the plasmid was in the V2 orientation under all growth conditions examined (Fig. 4, lane D). The different proportions (5 and 40%) of the pUST153 and pUST156 plasmids in the V2 orientation may reflect influences on Rci function of (i) the size of the DNA between the 19-bp repeats and (ii) the base composition of the DNA between the 19-bp repeats.

Growth of E. coli JM109 strains containing individual plasmids belonging to the pUST151-pUST153 series in tetracycline-containing media (also with IPTG) was examined (Fig. 5). In LB with tetracycline at a concentration of 15 μg/ml, both E. coli JM109/pUST151 and E. coli JM109/pUST153 grew well, although E. coli JM109/pUST153 was somewhat slower to enter the stationary phase than E. coli JM109/pUST151. Strain E. coli JM109/pUST152 did not grow at all, as expected, since the Tc’ gene cannot be transcribed from the lac promoter. When nalidixic acid (15 μg/ml) was added to the growth media in addition to the tetracycline and IPTG, E. coli JM109/pUST151 exhibited slight growth inhibition, but E. coli JM109/pUST153 did not grow at all. Again, the negative control (E. coli JM109/ pUST152) failed to grow. The Rci protein is a member of the λ integrase family of recombinases (5). The λ integrase does not act on DNA which is relaxed, and increasing the extent of DNA supercoiling is known to enhance λ integrase activity (5). Thus, increasing DNA supercoiling may be expected to enhance Rci activity. It is suggested, then, that nalidixic acid-induced DNA supercoiling increased Rci activity and that such an increase inhibited transcription of the Tc’ gene (see below).

To explore this possibility further, E. coli JM109 strains containing plasmids belonging to the pUST154-pUST156 series were grown in the presence of ampicillin and IPTG, and XylE levels were assayed. In a typical experiment, the positive control (E. coli JM109/pUST154) gave a XylE level of 40.2 U. The negative control value for E. coli JM109/pUST155 was 0.7 U (1.7% of the positive control value). The test strain, E. coli JM109/pUST156, gave a XylE value of 2.3 U (5.7% of the positive control value), even though the equilibrium level of a plasmid permissive for xylE transcription from the lac promoter was ca. 40% (Fig. 4). Again, this suggested that Rci-mediated inversion of DNA including the xylE gene inhibited transcription of the gene.

An explanation of these results is that while the relative proportions of the two possible orientations (orientations V1 and V2) remain constant regardless of the growth conditions, the time spent in the orientation permissive for transcription of the gene between the two 19-bp repeats does not. With plasmid pUST153, DNA between the 19-bp repeats exists 95% of time in the V1 orientation and 5% of the time in the V2 orientation (Fig. 4). The time spent in the V1 orientation (permissive for Tc’ gene transcription from the lac promoter) is sufficient to allow complete transcription of the Tc’ gene located between the 19-bp repeats. It is proposed that addition of nalidixic acid increases the rate of Rci-mediated inversion such that the time spent in the V1 orientation becomes inadequate to allow completion of transcription of the Tc’ gene. Bacteria harboring plasmids undergoing rapid inversion events are Tc’ because RNA polymerase molecules transcribing the Tc’ gene are proposed to depart from the DNA as an inversion event occurs. With plasmid pUST156, DNA between the 19-bp repeats exists 60% of the time in the V1 orientation and 40% of the time in the V2 orientation (Fig. 4). The time spent in the V2 orientation (permissive for xylE transcription from the lac promoter) is proposed to be insufficient to allow complete transcription of the xylE gene located between the 19-bp repeats before the DNA is switched back to the V1 orientation.

The bundle-forming type IVB pili of enterotoxigenic E. coli increase bacterial virulence by promoting bacterial aggregation (1), and it is thought that the type IVB pili of V. cholerae have a similar function (6). It is therefore reasonable to suggest that the bacterial self-association promoted by serovar Typhi PilV-free type IVB pili is important in vivo for enhancement of bacterial invasion of the cells of the human small intestinal wall. Rci-mediated inversion of DNA between the 19-bp repeats in the C-terminal region of the pilV gene is enhanced by conditions that increase DNA supercoiling (5), and transcription of DNA between the 19-bp repeats appears to be inhibited by inversion events, presumably because the RNA polymerase detaches from the DNA during inversion. Under conditions that favor DNA supercoiling (such as low oxygen tension in an anaerobic small intestine), it may be expected that pilV transcription will be ineffective. The pili therefore do not carry PilV proteins, and the bacteria self-associate in the manner of the ΔpilV mutant. Under reduced DNA supercoiling conditions, PilV proteins are made, and the bacteria are dispersed. This may be relevant for dissemination of the bacteria in the extrahuman environment. The Rci-mediated inversion in the shufflon region may therefore control bacterial association and disassociation. Rapid toggling of the rci switch may inhibit pilV transcription and thus PilV synthesis; lower inversion frequencies may permit PilV expression. This idea also provides an explanation for why serovar Typhi has only two 19-bp repeats, so that only two different PilV proteins are possible, while plasmids R64 and R721 have seven and six 19-bp repeats, respectively (2, 3). In R64 and R721, the various PilV proteins selectively recognize determinants in the outer membranes of
different enterobacterial species and are thus responsible for preferential selection of recipients in liquid mating (the conjugative R64 or R721 plasmids are transferred) (12). In serovar Typhi, the PilV proteins do not have this function. It is necessary only that a PilV protein be sometimes synthesized and sometimes not synthesized, so that bacterial self-association (by PilV-free pili) may be regulated. The minimum number of 19-bp repeats required for such control is two, the number which we found.

Type IVB pili of serovar Typhi may be important in typhoid fever pathogenesis. Serovar Typhi contains a type IVB pil operon that is absent in serovar Typhimurium, elimination of the pilS gene reduces the ability of serovar Typhi to enter human intestinal cells in vitro, and purified pre-PilS protein inhibits such entry by wild-type bacteria (15). In the work reported here, we found that the serovar Typhi type IVB pil pili mediate bacterial self-association, but only when the PilV proteins are made. In turn, PilV expression may be controlled by the rate of Rci-mediated shufflun inversion, which is ultimately influenced by the extent of DNA supercoiling. Under anaerobic conditions in the human small intestine clumps of serovar Typhi bacteria may be formed. We suggest that the bacteria on the exterior of such aggregations may then bind to the human intestinal cell membrane. The next step would logically be bacterial invasion of the intestinal mucosa, not by single bacteria but by the pilus-promoted bacterial aggregates. This proposed type IVB pilus-enhanced event in typhoid fever invasiveness cannot occur in (for example) serovar Typhi-Typhimurium infections of the human intestinal tract, and this may help explain why only serovar Typhi, among the hundreds of _Salmonella_ serovars known, causes epidemics of typhoid fever.

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